Cre mediated approaches to the role of Oct-4 in the mammalian germline

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Thesis presented for degree of Master of Philosophy
University of Edinburgh
2004

Acknowledgements

I would like to thank my supervisors, Austin and Jenny, and also Clare for their guidance and support throughout my MPhil.

In the lab, I would particularly like to thank Frances for teaching me outstanding laboratory etiquette and reinforcing the use of β-mercaptoethanol in the fume hood; Renee for moral support and creating general chaos and Joe for having an answer to virtually everything. In the mouse house thanks to Carol & Co for comprehending hectic breeding schemes and general mouse management. Also other ICSR students, Dean and Marios (now postdoc) for their chitchat.

Friend wise, it's a lot of Alisons - Ali B, Ali F, Ali W - for starters then Katie, Katrina and Sonia for coffee, drinking and dancing. Also thanks to Angela, Graeme, Helen, Rachel J, Rachel M and Sarah for their constant e-mails, telephone calls and for some highly memorable visits to Edinburgh.

Finally thanks to my parents, David and Fiona, and brother Don for doing their thing.

Abstract

Oct-4 is a member of the POU family of transcriptional regulators the majority of which are expressed during embryonic development and are involved in the regulation of cell fate decisions (Ryan *et al.* 1997). Previous research has demonstrated that Oct-4 is absolutely required for the maintenance of pluripotency, both *in vivo* and *in vitro* (Nichols *et al.* 1998; Niwa *et al.* 2000). Oct-4 is expressed by the pluripotent cells of the early embryo, and, it is also expressed during specification and development of the germ cell lineage and in developing oocytes (Yeom *et al.* 1996).

This project is concerned with investigating the consequence of inactivating Oct-4 during germ cell lineage specification/development and oogenesis using a Cre-loxP approach. To address this issue it is imperative that the Cre-loxP systems employed cause deletion of the floxed Oct-4 target gene in a spatially and temporally predicted manner with a high efficiency. To achieve targeted inactivation in primordial germ cells, the TNAP-Cre line was used (Lomeli *et al.* 2000). Adult mice that were expected to lack Oct-4 in their germ cells were fertile and E12.5 embryos had no overt defects in germ cell numbers in their genital ridges. Pups born from these animals exhibited a mosaic pattern of inheritance of the recombined/non-recombined allele indicating that Cre mediated deletion occurred post fertilisation in these pups and not as a result of deletion in the germ cells. No pups were born that could be attributed to originate from an Oct-4 null germ cell. Furthermore and unexpectedly, using dormant reporter lines ectopic TNAP-Cre activity was detected in preimplantation embryos as opposed to being confined to the germ cells later during development. To achieve targeted inactivation in oocytes during oogenesis, the ZP3-Cre line was employed (Lewandoski *et al.* 1997). ZP3-Cre mediated deletion was found to be specifically confined to developing oocytes and was detectable in primary oocytes onwards. Unexpectedly, pups born from females that theoretically lacked Oct-4 in their oocytes always inherited the recombined Oct-4 allele in a Mendelian fashion. This suggests that recombination at the floxed Oct-4 locus had occurred in the oocyte or possibly in the single cell embryo, raising the possibility that Oct-4 is not required in oocytes.

The complications that have arisen in using the TNAP-Cre expression line have detracted from addressing the original question of the role of Oct-4 in germ cells. Furthermore, since no reporter gene was activated on recombination at the floxed Oct-4 locus, the assessment of Cre activity relied on the activation of silent reporters which may not have necessarily reflected the activity at the floxed Oct-4 locus. Mosaic ectopic TNAP-Cre mediated deletion in the early embryo is likely to account for recombination at the floxed Oct-4 target allele since no Cre recombinase was detected in genital ridges from E12.5 TNAP-Cre$^{+/-}$ embryos.

Reporter line studies showed that ZP3-Cre had the expected expression pattern. In mutant animals it was not possible to deduce whether the oocytes were actually deficient in Oct-4 protein and given the fact that all ooctytes of the correct genotype had undergone recombination it was also conceivable that the timing of the excision may have occurred too late, i.e. post the burst of Oct-4 expression as oocytes exit meiotic arrest, oocytes being viable due to the presence of maternal Oct-4 mRNA. The reasons for late excision and lack of phenotype are discussed.

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bp	base pair
DE	distal enhancer
EC cell	embryonic carcinoma cell
ES cell	embryonic stem cell
ICM	inner cell mass
IRES	internal ribosome entry site
Kb	kilobase
ORF	open reading frame
PGC	primordial germ cell
PE	proximal enhancer
RA	retinoic acid
TNAP	tissue non-specific alkaline phosphatase
UTR	untranslated region
ZP3	Zona pellucida 3

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Chapter 1: Introduction

1.1 Oct-4: a class V POU domain transcription factor

Oct-4, alternatively know as Oct-3 or POU5F1, is a member of the class V POU domain transcriptional regulators. Oct-4 was originally identified as conferring DNA binding activity in extracts from embryonic stem (ES) and embryonic carcinoma (EC) cell lines (Lenardo *et al.* 1989; Okamoto *et al.* 1990). POU proteins recognise and bind a 8 basepair (bp) recognition element, referred to as the octamer sequence , that is found in upstream regions of a number of genes (Ruvkun *et al.* 1991). The Oct-4 protein is 352 amino acids and contains a conserved POU domain of ~150 amino acids, consisting of the POU homeodomain and POU specific domain connected by a linker region (Okamoto *et al.* 1990; Rosner *et al.* 1990; Scholer *et al.* 1990). Both the POU-specific domain and POU homeodomain are required to achieve high-affinity site-specific DNA binding to the target octamer sequence ATGCAAAT (Herr *et al.* 1988). The POU-specific domain and POU homeodomain are able to bind DNA independently (Klemm *et al.* 1996) suggesting that the linker region acts to increase the local concentration of DNA binding domains thus increasing the site-specific DNA binding (Ryan *et al.* 1997). POU domain proteins have been identified from a number of species and have been classified into seven classes based on the POU domain sequence and the linker region (Ryan *et al.* 1997). Studies both *in vivo* and *in vitro* have demonstrated that POU transcription factors regulate principle developmental processes during animal development (Ryan and Rosenfeld 1997. Members of class V POU domain proteins, to which Oct-4 belongs, are expressed during early embryonic development.

In the mouse Oct-4 is located on chromosome 17 and maps to a position within the t-complex that is within the major histocompatibility complex (MHC) (Scholer *et al.*

1990). Orthologues of Oct-4 have so far only been identified in mammals including human (Takeda *et al.* 1992), bovine (van Eijk *et al.* 1999) and a marsupial (Frankenberg *et al.* 2001) but none has been identified in avians such as the chicken (Soodeen-Karamath *et al.* 2001). In Xenopus, three POU proteins have been identified that have a degree of homology to mammalian Oct-4; XLPou-60, XLPou-25 and XLPou-91 (Hinkley *et al.* 1992). These three genes exhibit a sequential pattern of expression during early embryogenesis and may play critical roles in the determination of cell fate and regulation of cell proliferation (Hinkley *et al.* 1992; Whitfield *et al.* 1995). Human and bovine Oct-4 genes map to a region within the MHC. They have been localised to human chromosome 6 (Takeda *et al.* 1992; Krishnan *et al.* 1995) and to the bovine chromosome 23 (van Eijk *et al.* 1999). The Oct-4 orthologues exhibit considerable similarity in sequence, genomic organisation and map position. For instance murine Oct-4 is highly conserved in humans (87%) and cows (87.1%) in overall protein sequence identity. Furthermore, comparative analysis of upstream promoter sequences of human, bovine and murine Oct-4 gene revealed that there were four highly conserved regions of homology between these species suggesting similar translational control mechanisms (Nordhoff *et al.* 2001). Interestingly the human orthologue of Oct-4, unlike any of the other orthologues, generates two transcripts, possibly via two alternative splicing mechanisms or an alternative 1st exon usage, producing two proteins with differing N-terminal regions (Takeda *et al.* 1992). The functions conferred by these two splice variants remains unidentified though considering each variant has the same DNA binding activity but a differing activation domain this is likely to be functionally significant.

1.2 Expression pattern of Oct-4 during development

Expression of Oct-4 occurs early in mouse embryogenesis in the pluripotent lineage but is down-regulated as embryogenesis proceeds where expression eventually becomes confined to cells only of the germ cell lineage. The expression pattern of Oct-4 is schematically illustrated in Figure 1. In unfertilised oocytes both maternal Oct-4 mRNA and protein are present that are transported to the nucleus following fertilisation (Rosner *et al.* 1990; Scholer *et al.* 1990; Yeom *et al.* 1991; Palmieri *et al.* 1994). Significant but low levels of Oct-4 protein are detectable in 2- and 4-cell embryos (Palmieri *et al.* 1994). Zygotic Oct-4 expression is activated prior to the 8-cell stage and noticeably higher levels of Oct-4 protein are detected in all nuclei throughout the morula stage (Yeom *et al.* 1996; Palmieri *et al,* 1994). Prior to blastocoel formation in the compacted morula a cell fate decision occurs depending on the down-regulation or maintenance of Oct-4 expression. This brings about the first separation of the extraembryonic and embryonic lineages (Okamoto *et al.* 1990; Rosner *et al.* 1990; Scholer *et al.* 1990). The outer cells of the morula downregulate Oct-4 expression and differentiate to form trophectoderm which is required for implantation and placental development. The inner cells of the morula maintain Oct-4 expression, they remain undifferentiated becoming the inner cell mass (ICM) which gives rise to the embryo proper plus other extraembryonic tissues. Similarly *in vitro* when ES, derived from the ICM, or EC cells derived from mouse teratocarcinomas, were induced to differentiate Oct-4 mRNA expression is downregulated (Lenardo *et al.* 1989; Scholer *et al.* 1989). Both in culture and in the embryo expression of Oct-4 is associated with undifferentiated cell type.

The ICM gives rise to the epiblast (also referred to as the primitive ectoderm) and hypoblast (also referred to as the primitive endoderm). Oct-4 expression is only maintained within the epiblast (Rosner *et al.* 1990; Scholer *et al.* 1990; Yeom *et al.* 1990).

The hypoblast, that surrounds the blastocoelic surface of the epiblast, is the second extraembryonic lineage. Just prior to implantation the hypoblast differentiates further into two cell lineages; the parietal and visceral endoderm. Interestingly a transient increase in Oct-4 expression occurs in the hypoblast before differentiation to parietal and visceral endoderm, neither of which expresses Oct-4 (Palmieri *et al.* 1994).

During gastrulation most of the positional information concerning the body plan is laid down. Also at this stage in development definitive separation of germline and somatic cell lineages occurs. Oct-4 expression, originally observed throughout the epiblast becomes down-regulated in an anterior to posterior direction and Oct-4 expression becomes restricted to the germline (Scholer *et al.* 1990). The epiblast is a cup-shaped structure, a thickening at one region of the "rim" specifies the posterior end of the embryo and indicates the onset of gastrulation. Epiblast cells at the anterior and lateral edges enter this thickening, referred to as the primitive streak, and migrate between the epiblast and endodermal cells giving rise to mesodermal and endodermal tissues. Some epiblast cells at the proximal region become segregated to the extraembryonic mesoderm at the embryonic/extraembryonic junction. As gastrulation continues the primitive streak lengthens anteriorly. Simultaneously though, the embryo is rapidly enlarging giving the appearance that the streak retracts posteriorly.

On reaching day E8.5, Oct-4 expression is only observed in PGCs in the extraembryonic mesoderm at the base of the allantoic bud (Yeom *et al.* 1996). PGCs are first identifiable from mid-gastrulation by their strong alkaline phosphatase activity. From the allantoic bud the PGCs migrate to the gonadal anlagen and continue proliferating until E13.5 when they become germ cells. This halt in proliferation is coincident with the beginning of sexual differentiation. Embryonic germ (EG) cells derived from the *in vitro* culture of PGCs also maintain expression of Oct-4 (Labosky *et al.* 1994; Yeom *et al.* 1996).

Expression of Oct-4 during gametogenesis is down-regulated in a sex specific manner (Pesce *et al.* 1998). In male embryos, Oct-4 expression is maintained in postproliferative prospermatogonia. After birth Oct-4 expression continues in undifferentiated A spermatogonia, however on reaching spermatogenic maturation expression is downregulated completely. In female embryos, Oct-4 is expressed until E15.5 in oocytes until the zygotene/pachytene stage of meiotic prophase 1 is reached. 1-3 days after birth, Oct-4 is re-expressed in meiotically arrested developing oocytes at low levels (Pesce *et al.* 1998). In full grown oocytes, that are stimulated to complete meiosis there is a dramatic burst of Oct-4 expression (Pesce *et al.* 1998). This first occurs in oocytes at 12 – 14 dpp and thereafter in full grown oocytes selected to complete meiosis in the adult female (Pesce *et al.* 1998). The expression pattern of Oct-4 during oogenesis is depicted schematically in Figure 1.2.

A more detailed discussion of the specification and development of germ cells and oogenesis are found in sections 1.6 and 1.7.

Figure 1.1 Expression of Oct-4 in the mouse embryo during embryogenesis. Light blue indicates the presence of maternal Oct-4 mRNA, dark blue indicates the presence of Oct-4 protein: zygotic Oct-4 gene activation occurs prior to the 8 cell stage of the embryo.

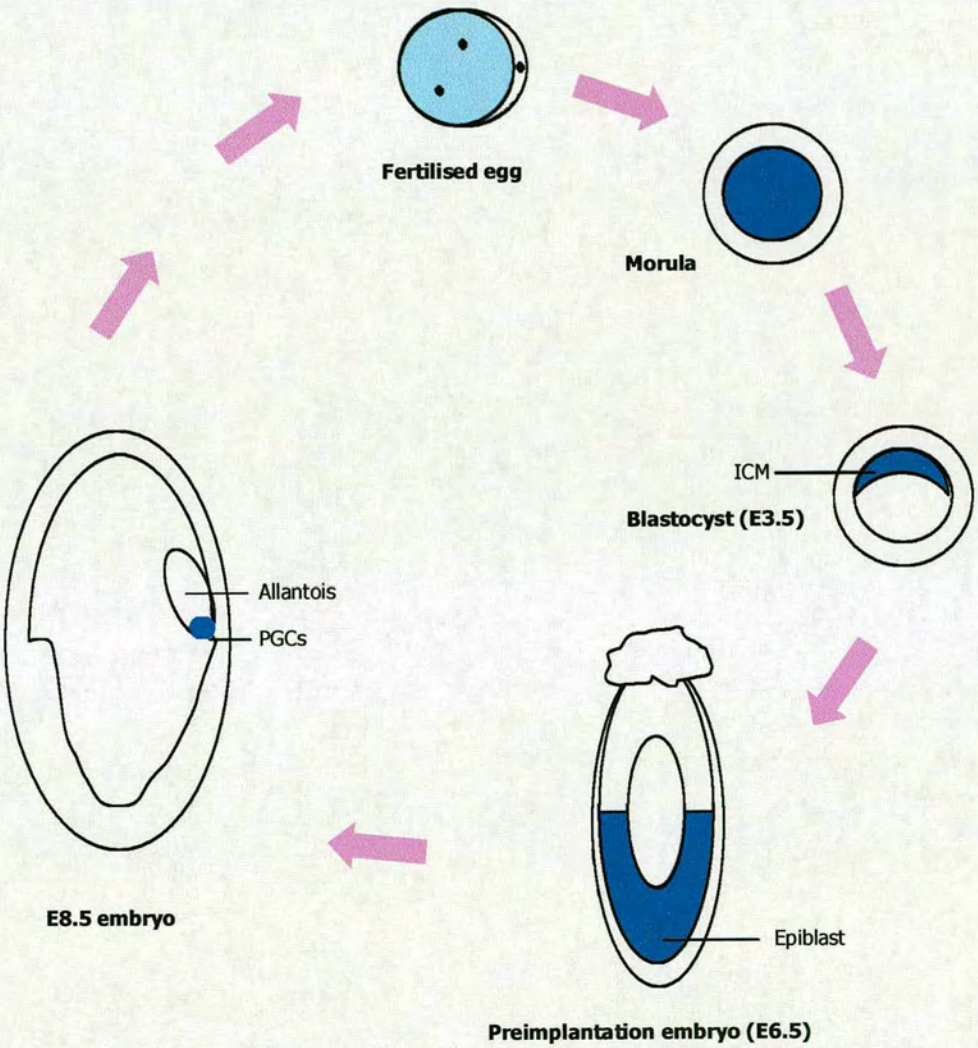
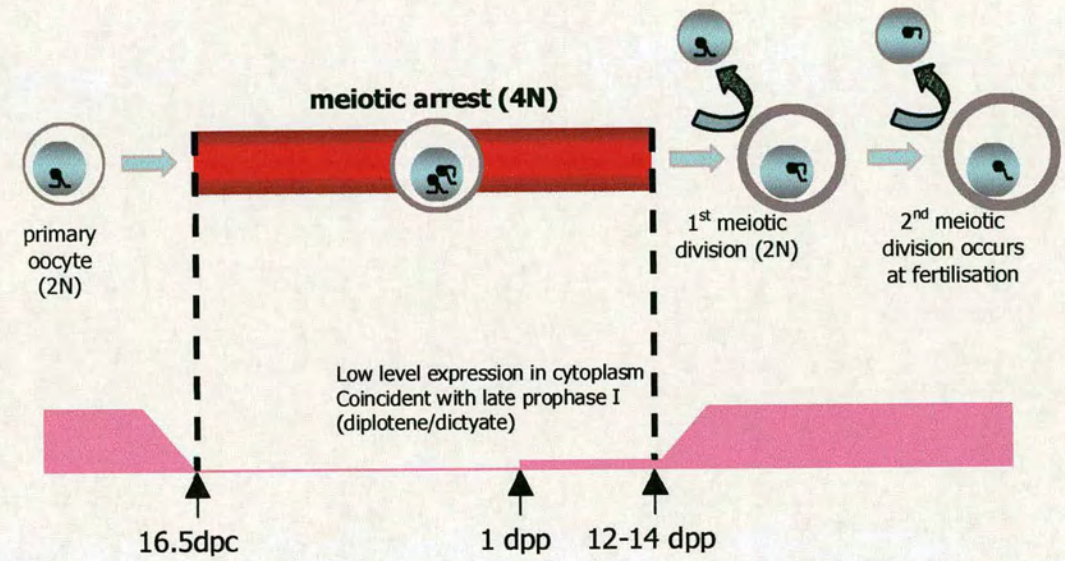


Figure 1.2 Expression pattern of Oct-4 during oogenesis.



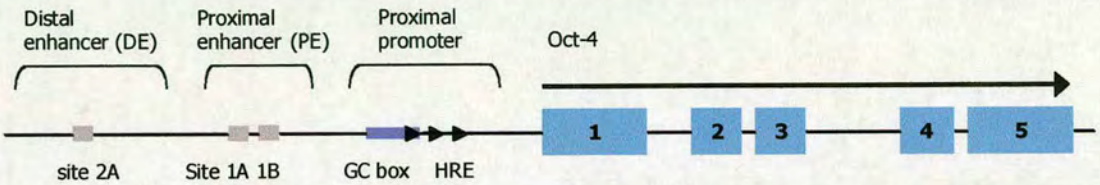
Down-regulation in all developing oocytes
Coincident with mid prophase I (zygotene/pachytene)

At 12-14 dpp first dramatic up-regulation in first cohort of developing oocytes stimulated to complete meiosis. This up-regulation is coincident with completion of prophase I and marks exit of growth arrest.

After this point those oocytes stimulated to exit growth arrest exhibit a dramatic up-regulation in Oct-4 expression. This occurs at different times for different oocytes subject to when a particular oocyte is selected to exit growth arrest and continues throughout the reproductive life of the mouse

1.3 Regulation of Oct-4 expression

1.3.1 Regulatory features of the Oct-4 5′ UTR



Distal enhancer: oocyte through to morula stage, ICM, PGCs, ES and EG cells

Proximal enhancer: epiblast, EC cells

Figure 1.3 Genomic structure of the Oct-4 gene. Oct-4 consists of five exons, depicted as blue boxes. The upstream regulatory regions depicted include the promoter, proximal enhancer and distal enhancer. Within these regulatory regions grey boxes represent factor binding sites. The activity of each of the enhancer elements in different cell types is indicated. HRE = hormone responsive element.

Transgenic analysis of Oct-4 expression in the developing mouse embryo using a LacZ reporter construct identified an 18kb region upstream of the Oct-4 open reading frame (ORF) sufficient to reproduce endogenous gene expression (Yeom *et al.* 1996). Further analysis of this region by deletion analysis identified three *cis*-regulatory regions necessary for endogenous gene expression. Located 5′, in close proximity to the Oct-4 transcription initiation site is the proximal promoter, upstream of which are located two enhancer elements; the proximal (PE) and distal enhancers (DE). The Oct-4 gene consists of five exons that encode a ~1.5kb mRNA transcript and the genomic structure is depicted schematically in Figure 1.3 (Okamoto *et al.* 1990; Okazawa *et al.* 1991; Yeom *et al.* 1991).

Within the Oct-4 proximal promoter are a series of overlapping DNA binding sites that are recognised by specific DNA binding proteins (Ovitt *et al.* 1998). These include a GC box, three repeated half sites of the consensus hormone response element (HRE) and a putative binding site for a nuclear orphan receptor (ELP) is present (Schoorlemmer *et al.* 1994; Sylvester *et al.* 1994). Oct-4 lacks a TATA box and Sp1 transcription factors are believed to play a role in initiating transcription from promoters lacking a TATA box. The lack of TATA box may be an important mechanism by which gene expression can be restricted in undifferentiated cells. In preimplantation embryos, ES and EC cells transcription of Oct-4 would depend on Sp1 binding, while the absence of the TATA box in differentiating cells would facilitate its down-regulation. *In vitro*, mutations in the GC box in ES and EC cells prevent Oct-4 expression, however *in vivo* expression of Oct-4 is unaffected possibly suggesting a degree of genetic redundancy within the Sp1 family members (Minucci *et al.* 1996). In another study, analysis of the activity of Oct-4 promoter GC box in Sp1 deficient ES cells showed that Oct-4 mRNA and protein levels were comparable to wildtype ES cells (Pesce *et al.* 1999). On further analysis of GC-box binding proteins in extracts from the $Sp1^{-/-}$ ES cells Sp3, another member of the Sp1 transcription factor family was identified and found to efficiently bind to the Sp1 site. These results may indicate that Sp3 may complement Sp1 function in ES cells and potentially other GC-box binding proteins may be involved (Pesce *et al.* 1999). Also present are three repeated half-sites of the consensus hormone response element (HRE) which resemble a retinoic acid response element (RARE) (Schoorlemmer *et al.* 1995). This site is recognised by several receptors including retinoic acid (RA) and retinoid X receptors (RXR) which belong to the steroid thyroid hormone receptor family.

The activity of the two enhancers is temporally regulated such that the DE drives expression in the morula, ICM and PGCs whilst the PE is active in the epiblast (Yeom *et al.* 1996) ES and EC cell lines derived from specific embryonic developmental stages also reflect this enhancer usage. In ES cells isolated from the ICM Oct-4 expression is

driven by the DE. In EC cells Oct-4 expression is driven by the PE. *In vivo* the exact timing of the switch in promoter usage is unknown, however it would appear that control of Oct-4 gene expression switches from the PE to the DE as PGCs are allocated to the extraembryonic mesoderm during gastrulation (Yeom *et al.* 1996).

Within the PE a *cis*-acting element that responds to RA repression is present, though it does not contain a recognisable RA receptor binding site (Okazawa *et al.* 1991). This element can be divided into two regions, 1A and 1B, only 1A remains occupied *in vitro* in undifferentiated ES and EC cells (Minucci *et al.* 1996). This site is also GC rich and related to the Sp1 binding sequence found in the promoter. The DE element contains a site called 2A that is an inverted version of site 1A in the PE. *In vivo* genomic footprinting analysis demonstrated that this site is also occupied in undifferentiated ES and EC cells (Minucci *et al.* 1996).

1.3.2 Retinoic acid (RA) and downregulation of Oct-4 expression

Considerable research has been carried out to investigate Oct-4 downregulation and cell differentiation using the ES and EC model systems. Treatment of ES or EC cells with RA induces differentiation and represses Oct-4 mRNA and protein expression (Lenardo *et al.* 1989; Scholer *et al.* 1989; Okamoto *et al.* 1990). Within the Oct-4 promoter-enhancer region are several RA-response elements (RAREs). Expression of Oct-4 is controlled by a cell specific enhancer, RARE1, located 1.2kb upstream of the initiation site this contributes to RA-mediated repression (Okazawa *et al.* 1991). RARE1 does not contain a typical recognition for RA receptors (RAR), however a RARE is also present in the Oct-4 promoter (RAREoct) and it would appear to be this site that mediates RA induced repression in RA differentiated EC cells (Pikarsky *et al.* 1994; Schoorlemmer *et al.* 1994). Within the Oct-4 upstream region are several target sequences for RA-mediated repression including the HRE which can bind members of

the nuclear hormone receptor subfamily (Ruvkun *et al.* 1991; Schoorlemmer *et al.* 1994; Sylvester *et al.* 1994). The orphan receptors ARP-1/COUP-TFII and EAR-3/COUP-TF1 are induced by RA treatment of EC cells and act as negative regulators at the Oct-4 locus (Ben-Shushan *et al.* 1995).

RA appears to cause loss of factor binding from the enhancer/promoter regions at the Oct-4 locus. For instance in RA treated ES and P19 EC cells factors are displaced from the proximal enhancer as shown by genomic footprinting (Minucci *et al.* 1996). Also, although there appears to be no RARE in the distal enhancer, factors binding to site 2A in the distal enhancer are also lost (Minucci *et al.* 1996). This may indicate that Oct-4 repression brought about by RA treatment *in vitro* occurs as a result of simultaneous coordinated loss of activating factors from the proximal and distal enhances. However, similar experiments performed on F9 EC cells did not give the same results; factor(s) were only lost from the proximal enhancer and not from the promoter or distal enhancer. This may reflect intrinsic differences between the two cell lines. F9 cells are though to represent a model system for differentiation of parietal and visceral endoderm(Hogan *et al.* 1981). Since Oct-4 expression decreases slowly even after 6 days of RA treatment this may reflect the *in vitro* situation where prolonged expression is associated with the differentiation of the parietal endoderm (Palmieri *et al.* 1994).

1.3.3 Methylation and downregulation of Oct-4 expression

Methylation is one is the molecular mechanisms that control gene expression (Bird 2002). During embryogenesis DNA methylation patterns undergo dramatic changes such that a global loss of methylation occurs in the mammalian preimplantation embryo prior to 16 cell stage. The DNA remains unmethylated until implantation, at which point an extensive wave of *de novo* methylation occurs and only those genes containing CpG islands escape, such as housekeeping genes (Bird 2002). Tissue

specific genes are subject to selective demethylation later during development (Bird 2002).

During mouse embryogenesis a specific restriction of Oct-4 expression occurs such that Oct-4 becomes confined to the pluripotent lineage; the germ cells. This is also reflected *in vitro* where Oct-4 is down-regulated in differentiating cells and maintained in undifferentiated ES cells. Differentiation of ES or EC cells with retinoic acid results in a dramatic reduction in Oct-4 expression and this is associated with loss of occupancy of transcription factors from the Oct-4 promoter and enhancer regions (Minucci *et al.* 1996). Furthermore, this is also associated with increased methylation and changes in chromatin structure in the upstream regulatory regions that include the promoter and proximal enhancer elements (Ben-Shushan *et al.* 1993). Recent work by Gidekel and colleagues has shown that *in vivo* the Oct-4 gene is unmethylated from the blastocyst stage and starts to undergo *de novo* methylation at E6.5 (Gidekel *et al.* 2002). This is of interest since Oct-4 gene remains unmethylated whilst the rest of the genome is undergoing *de novo* methylation and would indicate that there is a protection mechanism. Using a series of stable transfection assays in EC cells it was possible to identify a *cis*-specific demodification element within the Oct-4 proximal enhancer sequence. Mutations within this site resulted in methylation, thereby preventing transcription, and *in vivo* indicate that the proximal enhancer protects the DNA from *de novo* methylation in E6.25 epiblast cells. Both *in vitro* and *in vivo* it would appear that DNA methylation is sufficient to inhibit Oct-4 expression (Gidekel *et al.* 2002).

1.4 Role of Oct-4: target genes and functional partners

An insight into the biological roles of Oct-4 has been achieved both by targeted inactivation at the Oct-4 locus, analysing interactions with other regulatory proteins and identification of downstream target genes. Generation of a targeted null mutation

at the Oct-4 locus has demonstrated that Oct-4 expression is absolutely required for the identity of the pluripotent founder cell population in the mammalian embryo (Nichols *et al.* 1998). Embryos deficient in Oct-4 develop to form blastocysts, but cells allocated to the presumptive inner cell mass (ICM) region are not pluripotent and instead differentiate along the extraembryonic trophoblast lineage (i.e. differentiation along a somatic lineage).

Oct-4 can act both as a transcriptional activator or repressor either independently or synergistically with other factors (Pesce *et al.* 2001).

1.4.1 Oct-4 and functional partners

Oct-4 is able to activate target gene expression by interaction with co-activators or synergistic action with other factors. The adenoviral protein E1A was the first protein found to functionally interact with Oct-4 (Scholer *et al.* 1991). E1A and also other E1A-like proteins in stem cells enable distant dependent transactivation by bridging Oct-4 molecules to the basal transcription machinery. The E1A-Oct-4 interaction does not appear to require DNA binding on the part of E1A (Scholer *et al.* 1991).

Other Oct-4 target genes require the synergistic action of factors where both Oct-4 and the factor bind to each other and their target recognition sequences. For instance, an enhancer element (Curatola *et al.* 1990) located within the 5′ UTR of the fibroblast growth factor 4 (FGF-4) gene contains an octamer motif and an adjacent binding site to which the high motility group (HMG) transcription factor Sox-2 can bind. Oct-4 and Sox-2 bind cooperatively and activate transcription synergistically (Yuan *et al.* 1995). Both factors are absolutely required for enhancer activity since they appear to impart an allosteric change in each protein that is required for transcriptional activation of FGF-4 (Ambrosetti *et al.* 2000). Interestingly Sox-2 itself appears to be under the control of the

Oct-4/Sox-2 complex in ES cells (Tomioka *et al.* 2002). The Oct-4/Sox-2 complex also positively regulates Utf1, a transcription factor expressed in the ICM, by binding to an enhancer element in the 3′ UTR (Okuda *et al.* 1998; Nishimoto *et al.* 1999). Overall this indicates that Oct-4/Sox-2 transcriptional activation depends on reciprocal modelling of both proteins directed in a binding site specific manner. Another regulatory factor that binds to Oct-4 has been identified; Rox-1 (Ben-Shushan *et al.* 1998). Rox-1 in combination with Oct-4 or Oct-6 binds DNA recognition elements in the Rex-1 promoter to regulate transcription.

1.4.2 Oct-4 target genes

Oct-4 has been reported to bind to the regulatory regions of Rex-1 (Rosfjord *et al.* 1994; Ben-Shushan *et al.* 1998), FGF-4 (Schoorlemmer *et al.* 1991), the genes encoding human platelet-derived growth factor (PDGF) α receptor (Kraft *et al.* 1996), the α and β subunits of the human chorionic gonadotrophin (HGC) (Liu *et al.* 1997), a murine glucose transporter (Saijoh *et al.* 1996) and osteopontin (opn) (Botquin *et al.* 1998) .

Rex-1 and FGF-4 are expressed in ES and EC cells and expression of each is down-regulated on differentiation (Hosler *et al.* 1989; Schoorlemmer *et al.* 1991; Rosfjord *et al.* 1994). *In vivo* Rex-1 and FGF-4 are co-expressed with Oct-4 in the ICM (Rogers *et al.* 1991; Scherer *et al.* 1996). In Oct-4 homozygous null embryos which lack true ICM, trophoblast proliferation was not maintained and could only be restored by addition of FGF-4 (Nichols *et al.* 1998). Oct-4 directed gene expression of FGF-4 would appear to result in paracrine growth factor signalling from the pluripotent cell population to the trophectoderm (Nichols *et al.* 1998). Targeting inactivation at the Rex-1 locus to generated homozygous null ES cells resulted in ES cells that were unable to successfully differentiate into visceral endoderm on retinoic acid treatment alone

suggesting that Rex-1 plays a role in differentiation of this tissues type (Thompson *et al.* 2002).

Oct-4 has been shown to repress several target genes. Binding of the Oct-4 protein to the immunoglobin heavy chain enhancer in F9 EC cells resulted in repression of expression (Lenardo *et al.* 1989). Octamer motifs are found in the proximal promoters of the α and β subunits of the HGC genes that are required for implantation and maintenance of pregnancy (Liu *et al.* 1997). Each of the subunits were expressed as the trophectoderm began to differentiate, a time at which Oct-4 expression is down-regulated in the peripheral cells in the morulae. This indicated that HGC expression is repressed by Oct-4, and activated in its absence, its activation correlating to a time at which the embryo would require to implant and develop further. Oct-4 can act as a corepressor of FoxD3 and prevent activation of the promoters of the early endodermal transcription factors FoxA1 (previously HNF-3α) and FoxA2 (previously HNF-3β)(Guo *et al.* 2002). FoxA1, FoxA2 and FoxD3 belong to the Forkhead Box (Fox) family of transcriptional regulators. This family is strongly implicated in early embryonic lineage decisions, particularly the development of the endoderm and subsequent endodermal organogenesis. FoxD3 can activate the FoxA1 and FoxA2 promoters, however in the presence of Oct-4 activation is prevented due to Oct-4 physically interacting with FoxD3. Oct-4 alone cannot bind or activate the FoxA1 or FoxA2 promoters alone. Overall this data showed that Oct-4 can prevent ES cell lineage differentiation gene cascades by functioning as a corepressor of a lineage-specific transcription factor (Guo *et al.* 2002).

The majority of Oct-4 gene targets appear to have some direct role in differentiation but there are some exceptions. For instance, OPN protein is secreted by cells of the preimplantation embryo and binds to specific integrin subtypes and can modulate cell adhesion/migration Oct-4 and OPN are coexpressed in the preimplantation mouse

embryo and repressed during differentiation of ES and EC lines. An Oct-4 binding site has been identified in the OPN gene (Botquin *et al.* 1998). In the embryo OPN mRNA is found from in the epiblast and hypoblast. Oct-4, though it becomes restricted to only the epiblast there is a transient increase in the hypoblast as it begins to differentiate and migrate along the surface of trophectoderm. This overlapping expression in the premigratory endodermal cells suggested that Oct-4 is involved in the regulation of OPN expression and that OPN is involved in cell migration. Interestingly there is a Sox recognition sequence within the OPN promoter, but binding of Sox-2 appears to repress Oct-4 mediated activation at the OPN locus (Botquin *et al.* 1998). The reasons for these opposing actions remain unclear and require further characterisation (Ovitt *et al.* 1998).

1.5 Role of Oct-4 and cell potency

Oct-4 would appear to play an essential role in maintaining the pluripotent state of a cell, and potentially in the specification of the germ cell lineage. Expression of Oct-4 occurs during early mouse embryogenesis and Oct-4 expression is confined to undifferentiated or differentiating cells of the embryo. Targeted inactivation at the Oct-4 locus demonstrated that Oct-4 is absolutely required for the identity of the pluripotent founder cell population (Nichols *et al.* 1998). This and other studies suggest that the "decision" leading to Oct-4 expression or repression in a totipotent cell is one of the first events deciding whether a cell remains pluripotent or enters a somatic differential pathway (Nichols *et al.* 1998). Furthermore, it has been demonstrated that the precise level of Oct-4 governs three distinct cell fates (Niwa *et al.* 2000). In ES cells a critical level of Oct-4 expression is required to maintain a self-renewal capacity. Repression of Oct-4 expression directs dedifferentiation to trophectoderm, and elevation of expression directs differentiation along a primitive endoderm and mesoderm fate (determined by the presence of diagnostic markers for endoderm and mesoderm).

During gastrulation Oct-4 expression is progressively down regulated in an anterior to posterior manner and expression becomes confined to the germ cells of the embryo (Rosner *et al.* 1990). The role of Oct-4 in primordial and maturing germ cells has not been elucidated thought it has been speculated that Oct-4 is required for maintaining totipotency (Pesce *et al.* 2000). Prior to gastrulation, primordial germ cell (PGC) precursors are found within the proximal region of the epiblast. On formation of the primitive streak pluripotent cells within the epiblast region migrate to the extraembryonic mesoderm, some of which acquire a PGC fate. One hypothesis suggested for the allocation of germ cells in this manner is that this migratory mechanism may reflect the requirement for germline competent cells to avoid signals that drive somatic differentiation; instead maintaining a totipotent phenotype (i.e. maintaining Oct-4 expression) (Pesce *et al.* 1999). Furthermore expression of Oct-4 in the epiblast relies on the activity of the proximal enhancer, whilst expression in germ cells relies on the distal enhancer (Yeom *et al.* 1996). This enhancer switch could ensure that Oct-4 expression is maintained throughout the epiblast until specification of the germ cells. At this point the proximal enhancer is then switched off, whilst Oct-4 expression is maintained by the distal enhancer in germ cell precursors that must remain totipotent.

Expression of Oct-4 in PGCs persists until the onset of maturation in oogenesis and spermatogenesis; a stage coincident with entry to the meiotic cycle and coincident with meiotic arrest (Pesce *et al.* 1998). This may indicate that Oct-4 downregulation at this point may be one of the factors involved in determining commitment to meiosis. Further analysis of the maturation stages of oocytes has revealed that Oct-4 is upregulated dramatically in oocytes as they exit meiotic arrest and complete meiotic prophase I possibly suggesting subsequent roles of Oct-4 in oocyte growth and/or the acquisition of meiotic competence.

1.6 Specification and development of the germ cell lineage in the mouse

1.6.1 Specification of the germ cell lineage

In the mouse, unlike *Drosophila melanogaster* and *Caenorhabditis elegans*, development of the germ cell lineage is not a result of localisation of maternal components in the early embryo (Wylie 1999). Instead, beginning at approximately E6, cells derived from the proximal epiblast migrate into the extraembryonic mesoderm, a subset of which differentiate to form a precursor pool of approximately 50 primordial germ cells (PGCs) (Lawson 1994; Tam *et al.* 1996). PGCs are first detected at E7 - 7.25 as a population of alkaline phosphatase positive cells at the base of the allantois (Ginsburg et al. 1990) and have been observed *in vivo* using GFP under the control of the Oct-4 promoter (Yeom et al. 1996; Anderson et al. 2000). In the mouse, unlike in other organisms there is no evidence of maternally deposited germ plasm inherited by the germ cells (Zernicka-Goetz 1998; Ciemerych et al. 2000). In *Drosophila*, zebrafish and *Xenopus*, vasa and nanos are expressed in the early germ cells, but in the mouse these markers are not expressed until much later when the germs cells have completed their migration (Tanaka et al. 2000; Toyooka et al. 2000).

Fate determination in the epiblast is dependent on cell position suggesting the influence of local signaling factors. In the case of germ cells, heterotopic transplantation experiments in a E6.5 mouse embryo showed that distal epiblast cells when transplanted to the proximal region had a germ cell fate (Tam *et al.* 1996). Likewise when proximal epiblast cells were transplanted to the distal region they gave rise to distal cell fates. This would suggest that at E6.5 the tissues surrounding the proximal epiblast mediate a signal that induced germ cell fate.

Bone morphogenic proteins (BMPs) have been shown to be required for extraembryonic ectoderm to regulate the formation of the germs cells and related lineages. BMP4 and BMP8b, members of the transforming growth factor type β superfamily (TGFβ) of growth factors, have overlapping expression patterns in the extraembryonic ectoderm. Bmp4 homozygous null mutants completely lack PGCs and an allantois (Lawson et al. 1999), whereas Bmp8b homozygous mutants exhibited a severe defect in germ cell numbers, or none at all and poor development of the allantois (Ying et al. 2000). Similar phenotypes were also observed in SMAD1 and SMAD5 homozygous mutant embryos. This is perhaps not surprising since SMADs have been demonstrated to be the downstream signaling mediators for BMPs (reviewed in von Bubnoff *et al.* 2001). SMAD5 signaling appears to play a more general role in PGC generation and localisation whereas SMAD1 signaling is absolutely essential for the initial commitment of cells to the germ cell lineage (Chang *et al.* 2001; Hayashi *et al.* 2002).

Recent work has further investigated the related roles of BMP4 and BMP8b in PGC specification. Both BMP4 and BMP8b mutant animals exhibit a dose dependent reduction in germ cell number depending whether the animal is heterozygous or homozygous for the mutation. To elucidate whether BMP4 and BMP8b functioned as homodimers or heterodimers epiblasts of E6.0-6.25 embryos were co-cultured with BMP4 and BMP8b proteins expressed by COS cells allowing investigation into their effect on PGC induction. The results showed that BMP4 and BMP8b homodimers alone were unable to induce PGCs whereas they could in combination, suggesting that signaling via two BMP pathways are simultaneously required for PGC specification (Ying *et al.* 2001). Furthermore, the PGCs defects in the BMP4 homozygous mutant could not be rescued by BMP4 homodimers but the defect in the BMP8b mutant could be rescued by the presence of BMP8b homodimers indicating that BMP4 proteins are required for epiblast cells to gain germ-line competency prior to the synergistic action

of BMP4 and BMP8 (Ying *et al.* 2001). BMP4 is also expressed in the extraembryonic mesoderm and is also required for allantois development and the location and survival of PGCs (Fujiwara *et al.* 2001). Taken together, these results appear to suggest that BMP4 has two roles; it is firstly involved in the establishment of precursors from the epiblasts and the allocation of these precursors to the PGC or allantois lineages and secondly in the differentiation and survival of these cell types once they have been specified. Furthermore *in vitro*, treatment of epiblast cells from E5.5 – E6.0 embryos with BMP4 was sufficient to induce a PGC phenotype (Pesce *et al.* 2002).

1.6.2 Germ cell migration in the mouse

Germ cells in the mouse are specified in the posterior rim of the epiblast by local signaling and from here they migrate to an extraembryonic structure, the allantois, at E7.5. From the allantois, the germ cells migrate to the genital ridges via the hindgut (reviewed in Wylie 1999). Using a transgenic mouse line expressing GFP under a truncated Oct-4 promoter permits visualization of living germ cells *in vivo* and has begun to address the behavior of germ cells during the migratory process. Time lapse analysis showed that PGCs actively migrate from the time they exit the posterior primitive streak and enter the allantois (Anderson *et al.* 2000). Confocal microscopy in conjunction with time lapse imaging showed that at E9.0 the germ cells moved rapidly but are confined to the hind-gut (Molyneaux *et al.* 2001). Subsequently, at E9.5, the germ cells exited the hindgut and invaded the body wall prior to the mesentery forming; however, this movement does not appear to be directed towards the sites of the prospective genital ridges. Germ cells only begin to show directional movement towards the genital ridges at E10.5 and by E11.5 the majority of germ cells have colonised the genital ridges (Molyneaux *et al.* 2001). Experiments in culture have shown that germ cells move preferentially towards the genital ridges suggesting that this tissue sends out an attractive signal (Godin *et al.* 1990). *In vivo* it appears that any germ

cells that remains behind in the hind-gut mesentery do not migrate directionally any further and are lost , possibly by apoptosis (Molyneaux *et al.* 2001).

Germ cells take from E7.5 and E10.5 embryos have distinct migratory morphologies. They often have a tear drop shaped appearance and/or have pseudopodia extending from one germ cell to the next. During the migratory process, the germ cells do not migrate independently and instead associate with one another via cytoplasmic processes that form an extensive interlinking network (Gomperts *et al.* 1994). Post-migratory germ cells in the genital ridges are found in tight clusters without any pseudopodia. Both of these processes are also observed *in vitro*; on disaggregating and culturing germ cells from embryonic tissues on mouse embryonic fibroblasts, the germ cells first extend processes that contact other germ cells then segregate themselves from other cell types by forming tight non-motive invasive clusters of PGCs (Gompert *et al.* 1994)

Several genes are known to be required for germ cell migration/survival. Recently the mouse germ cell deficient (gcd) mutation (Pellas *et al.* 1991; Duncan *et al.* 1995), characterised by reduced numbers of PGCs and adult sterility was found to be attributed to partial deletion of the proliferation of germ cells (pog) gene and results in reduced PGC proliferation (Agoulnik *et al.* 2002). Mice lacking an RNA recognition motif/ribonucleoprotein-type RNA binding protein, TIAR, that is highly expressed in germ cells fail to develop spermatogonia or oogonia (Beck *et al.* 1998). This is due to a severely reduced survival rate of migrating PGCs and in mutant animals none are present in the genital ridges at E13.5. The exact role of TIAR in promoting PGCs survival in the genital ridge is unclear, however it has been hypothesised that TIAR may play a role in signaling or to play a role in RNA stability of growth factors involved in promoting the survival and proliferation of PGCs (Beck *et al.* 1998). These include steel factor, LIF, basic fibroblast growth factor and their respective receptors.

Two of the most well studied and characterised genes know to affect germ cells are dominant white spotted (W) and steel (Sl) (Godin *et al.* 1991; Buehr *et al.* 1993). Mutations in either of these genes have severe consequences since PGC are unable to populate the genital ridges. This is due to the cells being unable to proliferate and their migration is also affected. W and Sl encode the c-kit tyrosine receptor, expressed by germ cells, and the c-kit ligand stem cell factor (SCF), expressed in the mesentery, during germ cell migration. Migrating germ cells that stray from the Sl expression migratory pathway die, possibly due to apoptosis (Keshet *et al.* 1991; Motro *et al.* 1991). *In vitro* studies have confirmed the role of W and SL in culture but also suggest addition factors are likely to be involved. Germ cell isolated from genital ridges and cultured on a mouse embryonic fibroblast (STO) cell line require the presence of SCF for their survival in this *in vitro* system (Dolci *et al.* 1991; Godin *et al.* 1991). The rate of proliferation can also be increased by the addition of LIF (Matsui *et al.* 1991). The simultaneous addition of these factors appear to suppress apoptosis *in vitro* (Pesce *et al.* 1993).

Adhesion molecules are thought to play an essential role during germ cell migration and development. Cadherins have been proposed as the main candidate molecules that govern the germ cell interactions due to the fact that in both male and female mice PGCs express P- and E- cadherins during and after migration, and N-cadherin in post migratory PGCs (Wu *et al.* 1993; Lin *et al.* 1996; Packer *et al.* 1997). Blocking E-cadherin interactions using an anti-E-cadherin blocking antibody on E10.5 slice cultures caused defects in germ cell migration in the explants (Bendel-Stenzel *et al.* 2000; De Felici 2000; Di Carlo *et al.* 2000). P-cadherin targeted knockout mice are fertile and there was no apparent germ cell migratory phenotype (Radice *et al.* 1997). Targeted mutations of N- and E-cadherin genes resulted in early embryonic lethality and as a consequence their role in germ cell migration cannot be analysed by this approach (Radice *et al.* 1997). A

second potential candidate for germ cell adhesion is integrin. The β1 subunit of intergrin appears to be required by germ cells during colonisation of the genital ridges. However, a number of other integrin subunits are expressed in germ cells including α3, α6, and αV and targeted deletion of these genes did not result in any apparent germ cell phenotype (Anderson *et al.* 1999).

1.6.3 Germs cells in the genital ridge

GCs in the mouse first enter the genital ridges between E10 – E11 as the actual ridges are developing. By E11.5 a distinct boundary is apparent between the genital ridge and the mesonephros making the entry of further germ cells unlikely. From the original pool of approximately 50 PGCs, there are some 12 successive doublings resulting in approximately 25 000 germ cells in each genital ridge by E13.5 (Tam *et al.* 1981).

On entering the genital ridge the germ cells continue proliferating but they undergo a marked change in phenotype. The germ cells remain positive for alkaline phosphatase activity but become more rounded in shape and are less motile on explantation (Donovan et al. 1986). A number of other germ cells markers also begin to be expressed. Stage-specific-antigen-1 is first evident in germ cells at E9.5 and expressed until E12.5 (Marani *et al.* 1986). Germ cell nuclear antigen is first detected at E11.5 in germ cells within the genital ridge and is expressed in both male and female germ cell lineages until they reach the diplotene/dictyate stage of the first meiotic division (Enders *et al.* 1994). The mouse homologue of Drosophila vasa (Mvh) is expressed in germ cells within the genital ridge, and in spermatocytes and round spermatids (Fujiwara *et al.* 1994).

1.7 Oogenesis and folliculogenesis in the mouse

Close coordination between the developing germ and somatic cells in the mammalian ovary is required for the generation of viable eggs. During the reproductive life of the female, cohorts of primordial follicles are recruited periodically into a growth phase that results in meiotic maturation and ovulation of mature eggs into the oviduct in preparation for fertilisation.

1.7.1 Folliculogenesis and oogenesis are coupled processes

During folliculogenesis, the oocyte grows as the surrounding granulosa cells proliferate and differentiate. Completion of maturation and the first meiotic division occurs as the oocyte is ovulated into the oviduct in preparation for fertilisation. This process begins in the late stages of embryonic development when the primodial germ cells entered meiosis and arrest at the end of prophase I (dictyate in mice). These dictyate oocytes in the neonatal ovary subsequently become surrounded by a layer of flattened pre-granulosa cells, which in turn, are surrounded by a basal lamina to form primordial follicles where the oocyte itself has a diameter of approximately 12 - 15μm (Hirshfield 1991). This pool of primordial follicles represents the entire component of germ cells available for reproduction after birth.

After birth cohorts of primordial follicles are periodically recruited to enter a three week growth phase. This is an ongoing cyclic process and continues throughout the reproductive life of the mouse. During this time the maturing oocytes grow in diameter from 12-15 to 75-85 μm. Concomitant with the growth of the oocyte, the surrounding granulosa cells become cuboidal in shape forming primary follicles. The granulosa cells continue to proliferate further, forming a pre-antral follicle that consists of multiple layers of granulosa cells surrounding the oocyte within the follicular

basement membrane. The transition from pre-antral to an antral follicle is brought about by a surge of gonadotrophins, follicle-stimulating-hormone (FSH), that occurs after puberty (Kumar *et al.* 1997). Oocytes within large antral follicles remain arrested due to a continued interaction with the surrounding granulosa cells (Bornslaeger *et al.* 1986). Large antral follicles are approximately 600μm in diameter and the oocyte within has a diameter of 75 – 85 μm.

Ovulation occurs in response to a surge of luteinising hormone (LH) and this marks the end of folliculogenesis (Lee *et al.* 1996). At ovulation full grown oocytes complete their first meiotic division, extrude the first polar body and become arrested at metaphase II (Richards *et al.* 2002). Noticeably, there is a considerable reduction in the number of follicles that ovulate compared to the number of primordial follicles originally recruited for growth. This is due to follicles undergoing atresia (Richards 1980; Hirshfield 1989). Atresia tends to occur at pre-antral and antral stages but can occur earlier in follicle development and appears to be intrinsic to the oocyte or occur as a consequence of granulosa cell death. The reasons for this remain unclear.

Folliculogenesis and oogenesis are tightly coupled processes that are coordinated via gap junction communication. Signals from granulosa cells regulate meiotic arrests, promote oocyte growth and assist resumption of meiosis and maturation (Epifano *et al.* 2002). Likewise, the oocyte secretes signals that induce follicle formation, promote granulosa cell architecture, regulate steriodogensis and maintain the structure of the developing follicle (Eppig 2001).

1.7.2 Oocyte-specific factors affecting folliculogenesis

Elucidating the critical events in ovarian development has been assisted by the identification of a number of genes that are exclusively expressed in the oocyte. The

gene identified so far appear to function as multipurpose factors throughout folliculogensis and/or early development.

Coordination of the expression of the three zona pellucida genes (Zp1, Zp2, Zp3) has been attribute to Factor In the Germline (FIGα), a germ cell specific basic helix-loop-helix (bHLH) transcription factor (Liang *et al.* 1997). Female mice that are null for FIGα are infertile (Soyal *et al.* 2000). In these animals, primordial follicles were not formed despite null oocytes becoming meiotically arrested at prophase I during embryonic oogenesis. The ovaries become devoid of oocytes within a week after birth and prior to this no ZP1, ZP2 or ZP3 transcripts are detected. This suggested that FIGα plays a regulatory role in the expression of two oocyte specific gene pathways; those that initiate oogenesis and regulate the three genes encoding the zona pellucida. Furthermore, the persistence of FIGα in the adult females suggested that this gene may regulate other additional pathways that are required for normal ovarian development.

On entering the growth phase of follicle development, oocytes secrete the Growth and Differentiation Factor 9 (GDF9) and Bone Morphogenic Protein 15 (BMP15). GDF9 belongs to the TGFβ family, and during folliculogenesis it is involved in the promotion of early granulosa proliferation and induction of differentiation of cumulus cells in the antral follicle (Elvin *et al.* 1999). Follicles in female mice deficient in GDF9 failed to grow beyond the primordial due to a block in follicle growth (Dong *et al.* 1996). Oocyte growth and zona pellucida formation occur normally, but other aspects of oocyte differentiation are abnormal. This data suggested that GDF-9, an oocyte-derived growth factor, was required for the development of the follicle itself (Dong *et al.* 1996) Bmp15, also know as Gdf9b, has a high degree of homology (52%) with GDF9 and is also involved in the stimulation of granulosa cell proliferation (Carabatsos *et al.* 1998; Dube *et al.* 1998; Otsuka *et al.* 2001) and appears to play a role in the regulation of FSH receptor transcripts (Otsuka *et al.* 2001). Female mice deficient for BMP15 are sub-fertile

with reduced litter sizes due to ovulatory defects (Yan *et al.* 2001). Furthermore, analysis of Bmp15$^{-/-}$ / Gdf9$^{+/-}$ and BMP15$^{-/-}$ / Gdf9$^{-/-}$ double mutants showed that BMP15 and GDF9 appear to act synergistically in ovarian function (Yan *et al.* 2001).

1.7.3 Oocyte maturation

Meiotic maturation

Acquisition of meiotic maturation competence by the oocyte occurs at two stages during growth. Firstly the germ cell gains the ability to undergo germinal vesicle breakdown (GVBD) progressing to metaphase I (Canipari *et al.* 1984; Chesnel *et al.* 1995), and then secondly progresses to metaphase II (Sorensen *et al.* 1976; Wickramasinghe *et al.* 1991). The resumption and completion of meiosis in the oocyte involves three factors; Maturation Promoting Factor (MPF), a proto-oncogene MOS and Mitogen Activated Protein Kinase (MAPK).

MFP activity appears to be a universal regulator of mitotic and meiotic cell cycles. MPF is a protein-serine/threonine kinase that consists of a catalytic subunit, p34^{cdc2}, and a regulatory subunit, cyclin B (O'Keefe *et al.* 1991). MPF activity rapidly increases upon the resumption of meiosis and continues to remain high until the metaphase/anaphase transition of meiosis I, after which it declines. Activity is increased again after polar-body extrusion and remains high while the oocyte is arrested in metaphase II. Activity of MPF during the cell cycle is controlled both by the phosphorylation of p34^{cdc2} p and by the association of cyclin. The cyclin B subunit appears to be the major determining factor that controls the timing of events that take place during meiotic maturation. Ledan et al (2001) demonstrated that the rates of cyclin B synthesis and degradation were essential for this timing mechanism (Ledan *et al.* 2001). They found that by injecting cyclin B transcripts with poly A tails of different lengths into oocytes resulted

in disruption of meiotic progression. $P34^{cdc2}$, the enzymatic/catalytic subunit of MPF also known as cyclin-dependent-kinase-1 (CDK1) is required for the resumption of meiosis after the temporary arrest at prophase I of meiosis. The phosphatases Cdc25a, Cdc25b and Cdc25c are activators of CDK1 and they regulate cell-cycle progression in mitosis and meiosis as well as the DNA damage response. Female mice null for Cdc25b are infertile due to their oocytes remaining permanently arrested in prophase I because of the inability to active MPF (Lincoln *et al.* 2002).

The mos proto-oncogene encodes a protein serine/threonine kinase and is required for the activation of MAPK. In the mouse oocyte, mos is required for metaphase II arrest and oocytes deficient in mos fail to arrest at metaphase II and undergo parthenogenetic activation (Colledge *et al.* 1994). Maturing oocytes from mos homozygous null mice mice fail to activate MAPK, while $p34^{cdc2}$ kinase activation remained normal until metaphase where it decreased prematurely (Choi *et al.* 1996). Furthermore, in mos null oocytes the first polar body appears to be abnormally large and does not always undergo normal rapid degradation, and instead cleaved further. This suggested that mos is not only a cytostatic factor but is also required for regulating the size and degradation of the first polar body (Choi *et al.* 1996). The MOS/MAPK pathways has also been shown to responsible for correct spindle formation at metaphase I and metaphase II and repression of DNA replication at the metaphase I – II transition (Verlhac *et al.* 1993; Colledge *et al.* 1994; Gebauer *et al.* 1994; Hashimoto *et al.* 1994; Araki *et al.* 1996).

Gap junctions

During folliculogenesis, the oocyte and the surrounding granulosa cells communicate with each other occurs via gap junctions. Gap junctions are located at sites of close cell apposition and they function as intercellular membrane channels that permit the free

flow of inorganic ions, second messengers and small metabolites (Nicholson *et al.* 1997). Gap junctions are formed from connexin proteins, of which there are 15 different family members. Different connexin genes have different patterns of expression; some of which have are expressed ubiquitously and other have a more restricted pattern (White *et al.* 1999). In the mouse CONNEXIN 37 (CX37) and 43 (CX43) are two of the key connexins for gap junction formation during folliculogenesis. CX37 is expressed by the oocyte througout folliculogenesis. Mice null for CX37 were infertile due to follicle development failing at the pre-antral to antral transition stage (Simon *et al.* 1997). These oocytes could be rescued by treatment with okadiac acid (a protein phosphatase inhibitor) resulting in the initiation of meiotic maturation; however, on removal of okadiac acid the oocytes rapidly revert to an arrested state (Carabatsos *et al.* 2000). Additionally, oocytes from these CX37 animals appear to have retarded growth in the later follicles, indicating that heterologous gap junction communication is not only required for oocyte cytoplasmic maturation but also completion of oocyte growth (Carabatsos *et al.* 2000). Unlike CX37, homozygous CX43 targeted mice died during late gestation due to an essential function in heart development (Reaume *et al.* 1995). Some oocytes were present in the neonatal ovary but there appeared to be a considerable reduction in the original germ cell numbers (10% of normal) (Juneja *et al.* 1999). To circumvent embryonic lethality neonatal CX43 null ovaries were cultured *in vivo* and grafted to a kidney capsule allowing ovaries to be studied for a time period equivalent to three weeks after birth (Ackert *et al.* 2001). The results showed that in the absence of CX43 folliculogenesis arrested prior to the follicles becoming multilaminar, the suggested that the CX43 gap junction plays an indispensable role both in germ line development and early folliculogenesis.

Zona pellucida

The zona pellucida is a glycoprotein based matrix synthesised by the oocyte that separates the oocyte from the surrounding granulosa cells. The zona pellucida is first detected in primary follicles as patches of extracellular matrix. As oocyte growth continues, the zona pellucida increases in width from 3μm in the secondary follicle to 7μm in an early antral follicle.

The zona pellucida is encoded by the products of three genes; Zp1, Zp2 and Zp3. Targeted inactivation at the Zp1, Zp2 and Zp3 loci have enhanced our understanding of the interaction of their gene products. Zp1 null mice were able to form a zona pellucida with ZP2 and ZP3, though it is structurally flawed, and mice were fertile albeit severely reduced due to precocious hatching of early embryos (Rankin *et al.* 1999). Mice that lack Zp2 are infertile but were able to form a zona matrix early in oogenesis but it was considerably thinner than normal and did not persist in ovulated eggs (Rankin *et al.* 2001). Mice lacking Zp3 are also infertile and completely unable to form any zona matrix even early in oogenesis like the Zp2 null line (Rankin *et al.* 1996). Oocytes isolated from each of the null lines can be matured and fertilised *in vitro* then cultured to the blastocyst stage. No live births resulted from the transfer of Zp2- and Zp3- null embryos to recipient females, though live pups were born from the transfer of Zp1- null embryos. These results suggested that only ZP3 is essential for formation of the zona pellucida. Furthermore, the morphological abnormalities observed during folliculogenesis of the Zp2 and Zp3 null animals adversely affect the developmental competence of the zona free oocyte. Importantly previous work has shown that artificial removal of the zona pellucida by biochemical means has no effect on development of the embryo (Naito *et al.* 1992). This may indicate that the

developmental defects associated with targeted inactivation of Zp2 and Zp3 are not only due to the physical absence of the normal zona pellucida matrix. ZP1, the least abundant zona pellucida protein, is thought to provide structural integrity to the zona pellucida since it is able to form inter-molecular disulphide bonds and cross link the ZP2 and ZP3 proteins (Green 1997). This would account for the functional properties of the zona pellucida remaining at low levels in Zp1 null oocytes but the structure being disorganized.

1.8 Fertilisation

For successful fertilisation of the ovulated oocyte the zona pellucida is required to mediate both the initial sperm-oocyte recognition and trigger the acrosome reaction. Originally it was believed that ZP3 was the primary sperm receptor that mediated recognition via the O-linked oligosaccharide side chains (Bleil *et al.* 1980; Florman *et al.* 1985). More specifically, the oligosaccharides implicated were the terminal α1,3 galactose (Bleil *et al.* 1988) and /or N-acetylglucosamine (Miller *et al.* 1992). Recently genetic studies have disputed this (Rankin *et al.* 1998). Furthermore, in mice that were unable to add the terminal α 1,3-galactose (Thall *et al.* 1995) or N-acetylglucosamine (Asano *et al.* 1997) residues due to a lack of the enzyme galactosyltransferase remained fertile.

The recognition between the ovulated oocyte and the sperm appears to require species specific recognition. For instance, human sperm will not recognize mouse oocytes. Considerable research has been carried to ascertain if ZP3 has any involvement in recognition between the sperm and the oocyte. In homozygous null ZP3 oocytes that express recombinant human ZP3 the mechanical structure of the chimeric zona pellucida appeared normal but there was no change in the specificity of sperm binding;

mouse sperm bind and fertilise the oocyte and human sperm cannot (Rankin *et al.* 1998). Additionally, the human ZP3 expressed in these mice had the same motility on a SDS-polyacrylamide gel as wildtype human ZP3 (64kDa) but this was distinctly different from the native mouse ZP3 protein (83kDa) (Rankin *et al.* 1998). This enforces the possibility that major post-translational modifications cannot convert the human ZP3 protein to mouse ZP3 protein. Clearly this cannot account for subtle post-translation modifications and may suggest that other proteins other than ZP3 are required for sperm recognition.

Immediately post fertilisation, the cortical granules that are located in the periphery enter the perivitelline space by exocytosis. This process results in biochemical modification of the zona pelludia and this appears to prevent other sperm from binding and fusing with the oocyte's plasma membrane. The modifications involve cleavage of ZP2 is into several peptides of approximately 21-31kDa that remain bound to each other via disulphide bonds (Bleil *et al.* 1981). Currently, it is unclear whether the cleavage of ZP2 is causative or reflective for preventing polyspermy and if there are other factors involved.

1.9 Gene expression during oogenesis and early embryogensis

During the growth phase of an oocyte, there are both qualitative and quantitative changes in key molecules involved in metabolism, structure and information. There are changes in cytoplasmic organelles such as the Golgi apparatus and mitochondria distribution (Gosden *et al.* 1997). In oocytes of small non-growing follicles very little is actually know about the transcriptional activity except that it is never completely silent. In contrast, once a follicle has been recruited for growth there is a dramatic up-regulation of transcription and translation that is maintained until the oocyte reaches its full size. In the mouse, RNA synthesis increases 300 fold to 15fg min^{-1} during peak

oocyte growth (Piko *et al.* 1982) and in the mature mouse oocyte there are approximately 0.3 - 0.5ng of RNA. On reaching fully size, mRNA synthesis in the oocyte virtually ceases and does not resume until 1 -3 days after fertilisation has occurred.

At fertilisation, both the male and female genomes are transcriptionally silent and the earliest stages of embryogenesis rely on maternal components previously synthesised by the oocyte. As the embryo develops the maternal components decay and there is a switch to transcription from the zygotic genome. Embryonic gene transcription is first detected at a low level in the male pronucleus in the late one cell embryo (Bouniol *et al.* 1995). Major activation of the embryonic genome does not occur until the two cell stage (Schultz 1993), at this point maternal mRNA has degraded to such an extent that only 30% of the total maternal mRNA remains. Given the key requirement of maternal proteins in early embryogenesis it is perhaps surprising that only a few maternal-effect genes have been identified in mammals. For instance, SPINDILIN (SPIN) is a maternal-effect protein expressed for only a short specific time period. SPIN, associated with formation of the first meiotic spindle, is detected in unfertilized oocytes and two cell embryos but not in eight cell embryos (Oh *et al.* 1997). Another example of a maternal-effect gene is Maternal Antigen That Embryos Require (MATER) (Tong *et al.* 2000). Female mice null for MATER are infertile; folliculogenesis, ovulation and fertilisation occur normally but embryos do not develop further then the two cell stage. In these embryos, transcription from the zygotic genome is initiated but become blocked at this point. Though the primer structure of MATER has been deduced, its' actual function during early embryogenesis remains unclear.

The control of timing of translation and degradation of stored maternal mRNA in the oocyte has been studied intensely. Within this population there are pronounced differences in the translation timing for different genes; this appears to be timed to

some extent by physiological requirements. For instance genes such as ZP3 are translated along with cellular housekeeping genes throughout oogenesis. Others such as *mos* and tissue-type plasminogen activator (tPA) involved in the cell cycle or at ovulation are not expressed until they are required later (Gosden 2002). The principle mechanism that controls masking-storage versus translational-degradation is selective cytoplasmic polyadenylation of mRNAs. Truncation of the pA tail masks the mRNA resulting in translational silencing, whereas extension produces activation. For instance, tPA normally has a tail length of ~ 250 pAs but when it is stored, and is transcriptionally inactive, the tail length reduces to ~ 40 residues (Meric *et al.* 1996). This transcriptional silencing is likely to be brought about by a trans-acting repressor protein, which if displaced by a competitor transcript results in rapid elongation of the tail (Stutz *et al.* 1998). A higher level of control of expression may be brought about by physical masking of the transcripts' regulatory elements by ribonucleoprotein (RNP) particles (Matsumoto *et al.* 1998) and/or interaction with cytoskeletal elements (Jansen 1999). These mechanisms have the effect as to uncouple the synthesis and translation of RNA into protein and are likely to contribute to the long half lives and rapid degradation of maternal mRNA in the oocyte.

1.10 The Cre-loxP tissue specific recombination system

1.10.1 Background

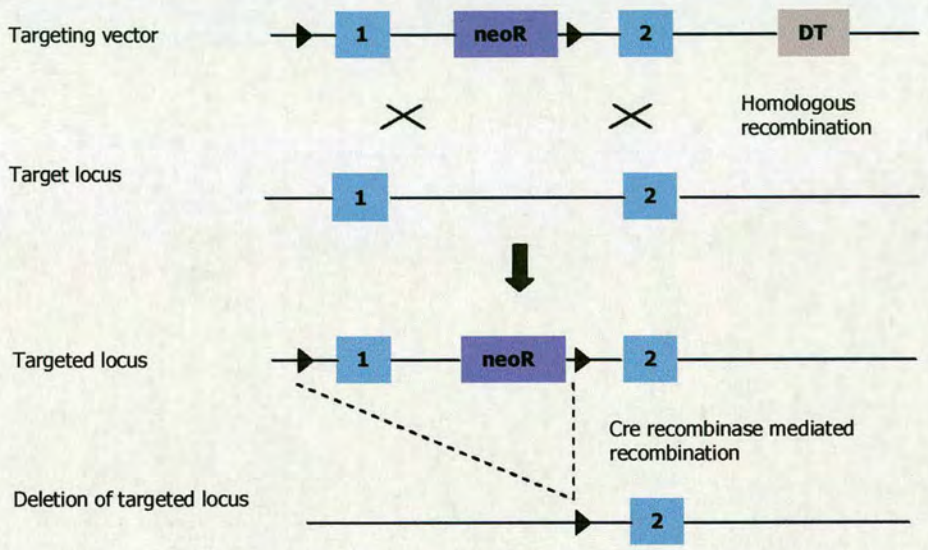


Figure 1.4 Schematic illustration of the principle of gene inactivation by expression of Cre recombinase on a loxP floxed target sequence. A targeted locus is generated by homologous recombination between a loxP containing target vector and the genomic locus where the boxed regions 1 and 2 represent exons. The target DNA sequence is floxed, expression of Cre recombinase results in excision of this DNA sequence including exon 1 and the neomycin resistance cassette. The presence of the neomycin resistance gene (neoR) and diphtheria toxin (DT) allows for detection of ES cell clones that have undergone correct homologous recombination.

Generation of mice with a targeted mutation of the gene of interest by homologous recombination has proven to be a highly successful approach for studying gene function (Brandon *et al.* 1995). A serious deficiency in this system concerns the time at which mice homozygous for the targeted mutation exhibit the phenotypic consequences. When mice heterozygous for the desired targeted mutation are crossed, homozygous offspring will suffer loss of gene function and hence loss of the corresponding protein expression from the time of conception throughout all tissues.

Due to this, mice homozygous for the mutation often die *in utero* where a gene product has a role(s) in embryonic viability (Lobe *et al.* 1998). Informative studies can be performed to identify why inactivation of a gene is lethal at that time, but functional analysis of the role of the gene later in development or in adulthood cannot be conducted. In other situations no apparent phenotype can be observed due to compensatory mechanisms, but this may in itself bring about unexpected phenotypes. A further complication is defining the gene function in a particular tissue. Where a gene is widely expressed non-tissue specific inactivation may ultimately result in a phenotype that is the combination of altering gene expression throughout the whole animal and not a specific tissue.

One way of avoiding the issue of embryonic lethality is to use an approach that targets gene inactivation in a specific temporal and spatical manner during development. Tissue specific gene inactivation is most commonly achieved by combining homologous DNA recombination and the action of Cre (Causes recombination events) site-specific DNA recombinase (Kuhn *et al.* 1997). Cre recombinase originates from the bacteriophage P1, and mediates recombination between specific 34bp DNA sequences named loxP sites. The loxP sequence consists of two 13bp inverted repeats interrupted by a 8bp palindromic sequence that defines the orientation of the site. Gene inactivation is achieved by deletion of a genomic region essential for gene function in a gene of interest. This is accomplished by introducing two loxP sites orientated in the same direction either side of the target genomic region (termed floxed). In the presence of Cre recombinase the floxed region is excised by Cre recombinase mediated recombination, as described in Figure 4. LoxP sites in opposing orientations result in inversion of the floxed sequences.

The Cre-loxP system has been used to achieve tissue specific gene activation. Tissue specific gene inactivation by the Cre-loxP system is achieved by crossing two strains of

transgenic mice, one expressing Cre recombinase under a tissue specific promoter and a second harbouring a floxed target gene (Kuhn *et al.* 1997). LoxP containing mice are generated by conventional homologous recombination gene targeting approaches. Tissue specific Cre recombinase expression is generally achieved by a 'knock-in' approach, involving introducing a Cre recombinase cDNA or gene immediately downstream of a specific endogenous promoter that has the required spatial and temporal expression pattern. This system has been shown to function well in ES cells and in transgenic mice (Muller 1999).

1.10.2 Specific spatial and temporal inactivation at the Oct-4 locus

Homozygous Oct-4 null embryos do not develop further than the blastocyst stage due to the absolute requirement of Oct-4 for the identity of the pluripotent founder cell population (Nichols *et al.* 1998). As a result it has not been possible to investigate the role of Oct-4 in the germ cell lineage by this conventional gene targeting approach. Within the ISCR there are a number of transgenic mouse lines that could be used to address this issue by achieving specific spatial and temporal inactivation at the Oct-4 locus using the Cre-loxP system, thus avoiding embryonic lethality. A floxed target allele of the Oct-4 gene (Niwa, H. unpublished) has been generated where loxP sites have been introduced flanking exons 2 and 5, this is illustrated in Figure 5. Two Cre recombination expression lines have been obtained where Cre recombinase is expressed by germ cells during their development and by oocytes during oogenesis; TNAP-Cre (Lomeli *et al.* 2000) and ZP3-Cre (Lewandoski *et al.* 1997) respectively. These are explained in more detail below and their genomic structures are illustrated in Figure 1.5.

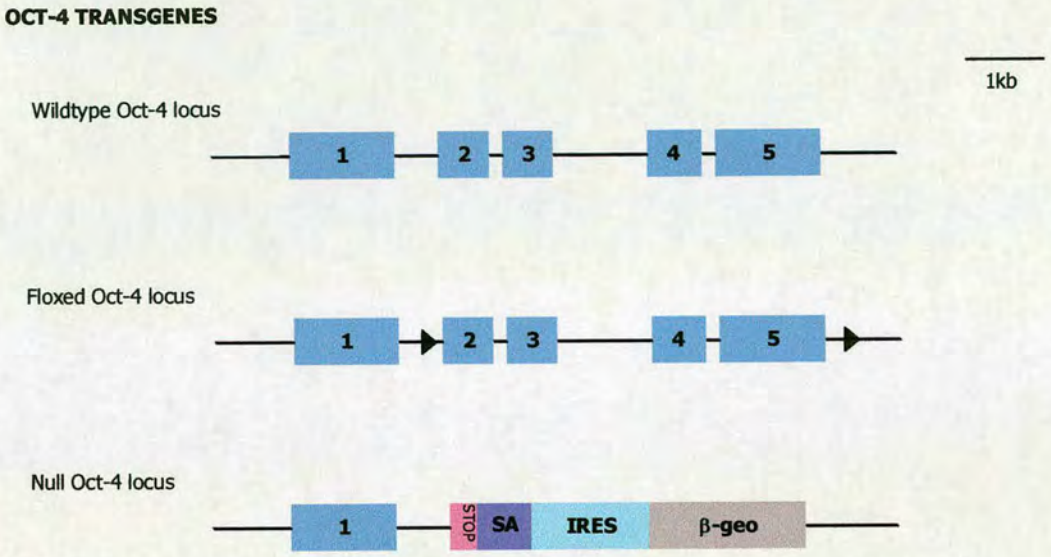
TNAP-Cre expression line

The expression pattern of tissue non-specific alkaline phosphatase (TNAP) is well-characterised (MacGregor *et al.* 1995). Targeting of a LacZ reporter construct to the TNAP genomic locus revealed that TNAP expression is first detectable at early E7 stage embryos in a small number of cells located within the extraembryonic mesoderm posterior to the primitive streak. Expression of TNAP is consistent with that published for germ cells; however at later stages of development expression is also detected in the intestine and spinal cord by E11.5. Furthermore, in E14 stage embryos expression is also detected within the developing skeletal system and the placenta. Published literature concerning the TNAP-Cre line (created by targeted integration of Cre into the TNAP locus) reports that when using the double Cre reporter line, lacZ/human alkaline phosphatase (Z/AP) (Lobe *et al.* 1999), approximately 60% of germ cells underwent recombination (Lomeli *et al.* 2000).

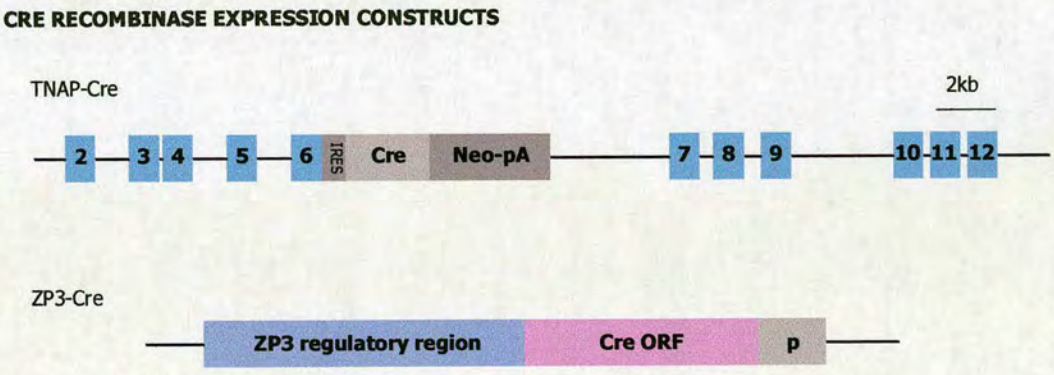
ZP3-Cre expression line

ZP3 is expressed in the zona pellucida of growing oocytes (Philpott *et al.* 1987; Dean *et al.* 1989). Experiments have been performed by Lira and colleagues who have identified the ZP3 regulatory region (Lira *et al.* 1990). A 6.5kb region within a 5′ region adjacent ZP3 was sufficient to drive expression of a luciferase reporter construct in the expected ZP3 expression pattern. Using this regulatory region, a ZP3-Cre line has been generated by random integration of the construct (Lewandoski *et al.* 1997). Lewandoski and colleagues reported that the ZP3-Cre resulted in Cre-mediated recombination apparently occurring in 100% of oocytes of a floxed target gene.

Figure 1.5 Structure of Oct-4 transgenes and Cre expression constructs



Oct-4 transgenes. The wildtype, floxed and null allele of Oct-4 are depicted above. Within the floxed Oct-4 locus, the location of loxP sites are indicated by the presence of filled triangles, the presence of Cre recombinase resulted in excision of this floxed region. Within the null Oct-4 locus a STOP-SA-IRES-β-geo cassette has been introduced that replaces exons 2-5. SA = splice acceptor, IRES = internal ribosome entry site.



Cre expression constructs. The genomic structure of TNAP-Cre (Lomeli *et al.* 2000) and ZP3-Cre (Lewandoski *et al.* 1997) are depicted above. For the TNAP-Cre expression construct a Cre recombinase expression cassette has been introduced by homologous recombination into the TNAP locus. Exons are depicted by blue open boxes, grey shaded boxes represent the exogenous DNA sequence. Within the grey shaded regions; IRES = internal ribosome entry site, Cre = Cre recombinase and Neo-pA = a neomycin selectable marker and polyadenylation signal. The ZP3-Cre cassette was generated by random integration. The 5′ region of the Cre ORF has been modified to optimise translational efficiency and includes a nuclear localisation signal. The polyadenylation signal (pA) was derived from the human β-actin gene.

1.11 Experimental strategy

In order to investigate the consequence of the inactivation of the Oct-4 gene in germ cells and oocytes adult mice were required that contained both the floxed Oct-4 target allele and the appropriate Cre expression line. Due to the nature of the Cre-loxP system and the problems sometimes associated with efficiency of excision at the floxed target locus it is general practice to have just one floxed target allele. To achieve homozygous inactivation at the Oct-4 locus a second Oct-4 line was used where Oct-4 has been inactivated by conventional gene targeting approaches (Nichols *et al.* 1998). The genomic structure of the Oct-4 null allele is illustrated in figure 1.5. A general breeding scheme to generate animals of an Oct-4 $^{-/loxP}$ TNAP- or ZP3-Cre genotype is illustrated in Figure 1.6. More detailed information regarding the breeding schemes for each of the Cre recombinase expression lines is discussed in each of the results chapters.

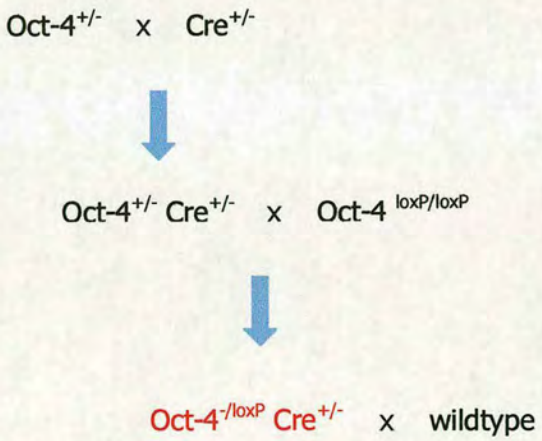


Figure 1.6 Transgenic mouse breeding scheme. This breeding scheme results in the generation of Oct-4$^{-/loxP}$ Cre mice (highlighted in red). Cre denoted here represents either ZP3-Cre or TNAP-Cre.

1.12 Aims and objectives

This aim of this thesis was to cause targeted recombination of a floxed Oct-4 allele generating Oct-4 null germ cells and developing oocytes using two specific Cre recombinase expression lines, TNAP-Cre (Lomeli *et al.* 2001) and ZP3-Cre (Lewandoski *et al.* 1997). Cre recombinase activity of each of the mouse lines was assessed by crossing to reporter lines. The consequence of Oct-4 inactivation in germ cells and oocytes was also investigated indirectly by analysing inheritance of the Oct-4 recombined allele and combining this with information gained from reporter line studies.

Chapter 2: Materials and Methods

2.1 Materials

Commonly used solutions were made according to Sambrook et al. (1989). All chemicals were of analytical grade and supplied by BDH or Sigma. Agarose (SeaKem LE agarose) for electrophoresis was obtained from Biowhittaker Molecular Applications (BMA). Restriction endonucleases were supplied by Roche and New England Biolabs. Synthetic oligonucleotides were supplied by Oswel DNA Service. Antibodies were obtained from BD Transduction Laboratories, Santa Cruz Biotechnology, Jackson ImmunoResearch Laboratories or donated by individuals (see Table 2.2).

Primers

Primer	Sequence (5'- 3')
Oct-4 null 1	TGACCGCTTCCTCGTGCTTTACG
Oct-4 null 2	GCCTTCCTCTATAGGTTGGGCTCC
Oct-4 null 3	GCTTATGATCTGATGTCCATCTCTGTG
Oct-4 floxed 1	GGATCCCATGCCCTCTTCTGGT
Oct-4 floxed 2	AACCCCAGGTGATCTTCAAAACAG
Oct-4 recombined 1	GGATCCCATGCCCTCTTCTGGT
Oct-4 recombined 2	GAGAAGAAGGCAGCCTTAGC
R26R 1	AAAGTCGCTCTGAGTTGTTAT
R26R 2	GCGAAGAGTTTGTCCTCAACC
R26R 3	GGAGCGGGAGAAATGGATATG
sGFP 1	CAGCAGCACGGGGCCGTCGCCGATGG
sGFP 2	CCGGGGTGGTGCCCATCCTGGTCGAG
TNAP-Cre 1	CACGTCGATGGCCGCTCTA
TNAP-Cre 2	TAAGGGCCAGCTCATTCCTCC
ZP3-Cre 1	TGATGAGGTTCGCAAGAACC
ZP3-Cre 2	CCATGAGTGAACGAACCTGG

Table 2.1 PCR primers for mouse/embryo genotyping.

Antibodies

Primary antibody	Source	Secondary antibody	Source
Oct-4 (N-19)	Santa Cruz Biotechnology, Inc	FITC donkey α-goat IgG	Jackson ImmunoReseach Laboratories
Oct-4 (Oct-3)	BD Transduction Laboratories	FITC rabbit α-mouse IgG	Molecular Probes
Stat-3 (C-20)	Santa Cruz	FITC goat α-rabbit IgG	Jackson ImmunoReseach Laboratories
Cre	Gift from G. Schutz, German Cancer Research centre	FITC goat α-rabbit IgG	Molecular Probes

Table 2.2 Details of primary and secondary antibodies.

Details of probes for *in situ* hybridisation

Probe	Linearise	Transcribed	Fragment size	Plamid	Source
Cre (antisense)	EcoRI	T3	850bps	pRCre	Elena Tzouanacou, ISCR.
Cre (sense)	ClaI	T7	850bps	pRCre	Elena Tzouanacou, ISCR.
Oct-4 (antisense)	EcoRI	T3	470bps	Oct-4 StuIStuI (AGS 122)	Ian Chambers, ISCR.
Oct-4 (sense)	XhoI	T7	470bps	Oct-4 StuIStuI (AGS 122)	Ian Chambers, ISCR.

Table 2.3 List of RNA probes

2.2 Embryology

2.2.1 Maintenance of animals

Mice were housed and bred within the animal unit at ISCR according to the provisions of the Animals Scientific Procedures Act 1986. Mice were maintained in a stabilised environment on a 12 hour light/12 hour dark cycle.

2.2.2 Recovery of embryos

To collect embryos at specific developmental stages, overnight matings were set up and the females examined for the presence of a vaginal plug on the subsequent morning. Noon on the day of finding a vaginal plug was designated E0.5. Embryos were dissected from the uterus in PB1.

PB1 media preparation (for 520mls), prepared by Frances Stenhouse.

sodium chloride	4.110g
potassium chloride	0.105g
sodium phosphate	1.500g
potassium dihydrogen orthophosphate	0.100g
glucose	0.520g
sodium pyruvate	0.023g
penicillin	0.030g
UHP water	513.5mls
$CaCl_2$ (stock 1.617g/100ml UHP water)	4.400mls
$MgCl_2$ (stock 3.131g/100ml UHP water)	1.600mls
0.5% phenol red	1.000mls

After the mixture was made, the solution was adjusted to a pH of 7.0-7.2 and sterilized through a 0.22 micron filter. 10% FCS was added to the final solution.

2.2.3 Induction of superovulation and collection of unfertilized oocytes

Five week old female mice were injected intraperitoneally (IP) with 0.1ml of pregnant mare's serum (PMS) (50IU/ml in 0.9% NaCl) followed by IP injection of 0.1ml human chorionic gonadotropin (hCG) (50IU/ml in 0.9% NaCl) 47 hours later. Superovulated unfertilised oocytes were dissected from the lumen of the oviduct and disaggregated from surrounding granulosa cells by treatment in hyluronidase solution (0.1% in HEPES-mCZB (H-mCZB) preparation, H-mCZB as described in Kimura and Yanagimachi (1995) then rinsed with PBS.

2.3 Molecular Biology

2.3.1 Mouse/embryo genotyping

Preparation of genomic DNA

Ear punches were taken from three week old pups by the animal house staff according to the provisions of the Animals Scientific Procedure Act 1986. For embryos, tissue samples were taken from either the embryo itself or from the yolk sac, then rinsed three times in PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2PO_4, 2mM KH_2PO_4 in dH_2O). Samples were incubated overnight in a shaking waterbath at 55°C in 50µl of embryo buffer (50mM KCl, 10mM Tris HCl pH 8.3, 2.5 mM MgCl2, 0.45% Nonidet P-40 (NP-40), 0.45% Tween-20) to which proteinase K had been freshly added (20µl of 20mg/ml proteinase K per 1ml of embryo buffer). Samples were boiled for 10 minutes at 100°C prior to centrifuging at 13K in a bench top centrifuge for 10 minutes. DNA samples were stored at -20°C.

Polymerase Chain Reaction (PCR) genotyping

Typically 5µl of genomic DNA was added to a reaction containing 5 units of Taq (QIAGEN), 1/10 volume of 10x QIAGEN PCR buffer, 0.2mM dNTPs and 100ng of primer per reaction with the total reaction volume being made up to 30µl with dH_2O. Primers sequences are detailed in table 2.1. PCR program cycles for genotyping each transgenic line are detailed in table 2.4. For the Oct-4 null PCR reaction, 400ng of primers 1 and 3, and 800ng of primer 2 were added to the reaction mix.

PCR reaction products were subjected to electrophoresis in 2% (w/v) agarose gel containing 0.5µg/ml ethidium bromide in 0.5 x TBE buffer (40mM Tris-borate, 2mM

EDTA). Table 2.5 details the sizes of the PCR reactions products. A 1kb DNA ladder (Gibco BRL) was used to estimate the size of the PCR products. DNA was visualised using an ultra violet (U.V) light emitting source.

	Oct-4 null	Oct-4 floxed/recombined	R26R
	95°C pause	95°C pause	94°C pause
Hot start	95°C 2min 45sec	95°C 2min 45sec	94°C 5min
Denature	94°C 15sec	94°C 15sec	94°C 45sec
Anneal	65°C 12sec } x34	60°C 12sec } x34	55°C 45sec } x35
Extension	72°C 1min	72°C 30sec	72°C 90sec
	72°C 10min	72°C 10min	72°C 10min
	4°C	4°C	4°C

	sGFP	TNAP-Cre	ZP3-Cre
	95°C pause	94°C pause	95°C pause
Hot start	94°C 3min	94°C 1min	95°C 5min
Denature	94°C 30sec	94°C 15sec	94°C 15sec
Anneal	55°C 30sec } x35	56°C 1min } x35	55°C 12sec } x34
Extension	72°C 1min	72°C 1min	72°C 1min
	72°C 3min	72°C 5min	72°C 10min
	4°C	4°C	4°C

Table 2.4. PCR programmes for mouse/embryo genotyping. For all PCR reactions except Oct-4 null, a Biometra UnoII machine was used. For the Oct-4 null PCR a Gene Amp PCR System 9700 (Applied Biosystem) machine was used. On reaching 4oC samples were either run out on an agarose gel or stored at -20°C.

	Oct-4 null	Oct-4 floxed	Oct-4 Rec.	sGFP	R26R	TNAP-Cre	ZP3-Cre
mut	545bps	358bps	500bps	520bp	250bps	262bps	389bps
wt	688bps	324bps	*	*	500bps	*	*

Table 2.5. PCR DNA product sizes for mouse/embryo genotyping. The * symbol indicates that no wildtype product was amplified in this reaction.

2.3.2 *Whole mount RNA in situ hybridisation*

Whole mount *in situ* hybridisation were carried as detailed previously (Wilkinson D. G. 1992). Embryo/tissues samples were fixed in 4% PFA overnight at 4°C. The samples were washed in PBS, then dehydrated through one change of 25%, 50% and 75% methanol:PBS, and finally in two changes of 100% methanol (5 minutes each wash, on ice). Embryos/tissue samples were then stored at -20°C.

Embryos/tissue samples were rehydrated through 75%, 50%, 25% methanol:PBS then washed twice in PBT (5 minutes each wash, room temperature). Embryos/tissue samples were incubated in 10μg/ml proteinase K in PBT; E7.5-8.5 embryos were incubated for 7.0 minutes, E10.5 embryos for 20 minutes and genital ridges from E12.5 embryos for 5 minutes. To inhibit the proteinase K activity the samples were incubated in glycine (2mg/ml in PBT), washed in PBT (2 x 5 minutes, room temperature) then refixed in 0.2% gluteraldehyde/4% PFA for 20 minutes. Samples were washed in PBT (2 x 5 minutes, room temperature) prior to incubating for 1 hour in prehybridisation solution (50% ultrapure formamide, 5 x SSC pH 4.5, 50μg ml heparin, 0.1% Tween 20) at 70°C. Denatured probe (1:100 dilution) was added to the hybridisation solution (as prehybridisation buffer but additionally containing 100μg/ml Torula yeast RNA, 100μg/ml of herring sperm DNA and probe). Embryo/tissue samples were incubated overnight in hybridisation solution at 70°C in a humidified chamber.

The following morning the embryos/tissue samples were washed twice in solution I (50% ultrapure formamide, 5x SSC pH 4.5, 1% SDS in dH_2O) for 30 minutes at 70°C, washed once in solution I:II (50% solution I: 50% solution II) for 10 minutes at 70°C then three times in solution II (0.5M NaCl, 10mM Tris pH7.5, 0.1% Tween 20 in dH_2O) for 5 minutes at room temperature. The samples were then incubated twice in RNAase A

(100μg/ml) in solution II for 30 minutes at 37°C. Washes were then performed once in solution II and solution III (50% ultrapure formamide, 2x SSC pH4.5 in dH_2O) for 5 minutes at room temperature prior to washing twice in solution III for 30 minutes at 65°C. Embryos/tissue samples were washed three times in TBST (10x 1.4M NaCl, 27mM KCl, 0.25M Tris pH7.5, 1% Tween-20 in dH_2O; use at 1x) for 5 minutes at room temperature, preblocked with 10% sheep serum in TBST for one hour at room temperature then incubated overnight at 4°C in a 1/2000 dilution of anti-DIG-AP FAB fragments (Boehringer) in TBST.

The next day the embryo/tissue samples were washed in TBST (6x 1 hour) with the last wash continuing overnight at 4°C. Subsequently the samples were washed three times in NTMT (100mM NaCl, 10mM Tris pH9.5, 50 mM $MgCl_2$, 0.1% Tween-20 in dH_2O) for 10 minutes at room temperature then incubated in 4.5μl NBT (Gibco/BRL), 3.5μl BCIP (Gibco/BRL)/ml NTMT in the dark at room temperature until colour development was observed. The reaction was stopped by washing three times for 5 minutes PBS containing 1mM EDTA and the samples stored in 0.01% azide in PBS at 4°C

All solutions were made up using 0.1% diethyl pyrocarbonate (DEPC) treated distilled water which was autoclaved prior to use.

2.3.3 Preparation and labelling of digoxigenin labeled antisense riboprobe

10μg of plasmid DNA was digested with the appropriate restriction endonuclease in a reaction mix containing 5 units of the appropriate restriction endonuclease (see Table 2.3), 1/10 volume of appropriate buffer and dH_2O to a final reaction volume of 200μl. This was incubated at 37°C for 2 hours. DNA was precipitated with 2½ volumes of absolute ethanol and 1/10 volume of 3M sodium acetate, then centrifuged at 13K in a bench top centrifuge for 10 minutes. The DNA pellet was washed in 70% ethanol,

centrifuged briefly, the supernatant was removed and the pellet air dried at room temperature. The DNA pellet was resuspended in 10µl of dH_2O. 1-3µg of DNA was added to 10µl 5x NTP mix, 5µl 10x transcription buffer, 50 units RNase inhibitor, 50 units of appropriate phage RNA polymerase and dH_2O to a final volume of 50µl. This was incubated at 37°C for 2 hours. Subsequently 20 units of RNAase-free DNAase was added to the reaction mix and incubated at 37°C for 15 minutes. To stop the reaction 4µl of 250mM EDTA was added, then the DNA was precipitated using 5.5ul of 4M LiCl and 165µl of absolute ethanol and left at -70°C overnight. The following day the sample was centrifuged at 13K for 15 minutes at 4°C. The supernatant was removed, the pellet was washed in 70% ethanol then air dried before being resuspending in 100µl of dH2O. 4-5µl of the probe was run out on 0.8% agarose gel to check for size and degradation. Prior to use an appropriate amount of probe was denatured at 95°C then immediately placed in ice before adding to prewarmed hybridization buffer.

All solutions were made up using 0.1% diethyl pyrocarbonate (DEPC) treated distilled water which was autoclaved prior to use.

2.3.4 Isolation of total RNA from animal tissues

Total RNA was isolated from animal tissues using the RNeasy Midi protocol (QIAGEN). In brief, the tissue was homogenized and the lysate centrifuged at 13 000 rpm for 5 minutes in a bench top microfuge. One volume of 70% ethanol was added to the supernatant, mixed by vortexing, then applied to the RNeasy midi spin column and centrifuged for 5 minutes at 13 000 rpm. The flow through was discarded and 3.8ml of buffer RW1 was added to the column followed by centrifugation for 5 minutes at 13 000 rpm. The flow through was discarded and 2.5ml of buffer RPE was added to the column followed by centrifugation for 2 minutes at 13 000 rpm. This was repeated a second time but the centrifugation step was carried out for 5 minutes. To elute the

RNA 150µl of RNase-free water was applied to the column and centrifuged for 3 minutes at 13 000 rpm. This was repeated a second time. Eluted RNA was stored at -20°C.

2.3.5 Reverse transcription PCR (RT-PCR)

First-Strand cDNA synthesis

First-Strand cDNA synthesis was performed as detailed in the SuperScript™ First-Strand Synthesis System for RT-PCR protocol (Invitrogen Life Technologies). In brief, to 1µg of total RNA, 50ng of random hexamers and 1mM dNTP mix were added to DEPC treated water to a total volume of 10µl. Sample were then incubated at 65°C for 5 minutes and incubated on ice for at least one minute. To each sample 2µl of 10X RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT and 1µl of RNaseOUT Recombinant Ribonuclease Inhibitor were added. Samples were incubated at 25°C for 2 minutes, followed by addition of 1µl (50 units) of SuperScript™ II RT followed by a further incubation at 25°C for 10 minutes. Samples were then incubated at 42°C for 50 minutes. The reaction was terminated by incubation at 70°C for 15 minutes. Synthesised cDNA was stored at -20°C.

PCR

5µl of the 1st strand reaction were used in a 30µl PCR reaction set up as described earlier in section 2.3.1. Conditions for the primer pair 187/188 for amplification of Cre were as described earlier in Table 2.4. Primers for β-actin were as follows: forward – GTGACGAGGCCCAGAGCAAGAG, reverse – AGGGGCCGGACTCATCGTACTC. For the β-actin PCR reaction a hot start at 94°C for 5 minutes was carried out, followed

by a cycle of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute repeated 20 times. Finally reactions were incubated at 72°C for 10 minutes and after reaching 4oC were either analysed or stored at -20°C. β-actin PCR reactions were performed on a Biometra UnoII machine.

2.4 Histology

2.4.1 Preparation of paraffin sections

Tissues samples were fixed in 4% PFA overnight at 4°C. The samples were washed in PBS and then dehydrated through 50%, 60%, 70%, 80%, 90% and 2 x 100% ethanol:PBS. Washes were carried out for 30 min at room temperature. Samples were cleared in xylene for 1 hour at room temperature before being placed in fresh in molten paraffin wax. The wax was changed three times and with 1 hour incubations. After cooling embedded tissue samples were stored at 4°C. 8μm sections were cut using a microtome (Anglian Scientific. 0325) and placed on poly-lysine coated slides (BDH).

2.4.2 Eosin staining

Wax sections were dewaxed in xylene for 10 min and rehydrated 100%, 95%, 70%, 50%, dH_2O ethanol:dH_2O (5 min each wash). Sections were stained for 4 min in 1% (w/v) eosin solution in dH_2O, washed briefly in dH_2O prior to dehydration through 50%, 70%, 95%, 100% ethanol:dH_2O (2 seconds each wash) then placed in xylene for 10 minutes. Stained sections were mounted under a coverslip in DPX mountant.

2.4.3 Staining embryos for ß-gal activity

Embryos (E3.5 to E12.5) were fixed for 30 min to 2 hours depending on their size in fixing solution (0.2% glutaraldehyde, 2mM MgCl, 5mM EGTA, 0.1M phosphate buffer pH7.3) at 4°C and washed three times for 20 minutes in wash solution (0.1M phosphate buffer pH7.3, 2mM MgCl, 0.1% (w/v) sodium desoxycholate, 0.02% NP-40, 0.05% BSA)

at room temperature. Staining was performed at 37°C in the dark in wash solution containing 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.H_20$ and 1mg/ml 5-bromo-4-chloro-3-indoly β D-galactopyranoside (X-gal) (Sigma) for 2 hours to overnight. X-gal was originally dissolved in dimethyl formamide at a concentration of 50mg/ml. Following staining, specimens were refixed overnight in fixing solution and stored at 4°C.

2.4.4 Staining of E12.5 genital ridges for alkaline phosphatase activity

Genital ridges were dissected from E12.5 embryos with fine forceps. The dissected tissue was washed twice in PBS for 15 minutes then fixed in 4% PFA for 2 hours. Fixed tissue was washed three times in PBT for 15 minutes and left overnight in 70% ethanol. The tissue was washed in dH_2O (3 x 15 min), incubated in Proteinase K solution (2.5μg/ml in dH_2O) for 3 minutes at 37°C then placed immediately in glycine (2mg/ml in dH_2O) for 15 minutes at room temperature. Prior to staining the tissue was washed in dH_2O (3 x 15 minutes). Staining was performed at room temperature for 10 minutes in the dark as described by Ginsburg (1990) using the staining solution (1% sodium 5'5'diethyl barbiturate, 0.12% $MgCl_2$, 0.02mg/ml α napthyl phosphaste, 1.0mg/ml fast red TR in dH_2O). Following staining, specimens were washed in dH_2O (2 x 10 minutes) and stored in 70% glycerol. All washes and incubations were performed at 4°C unless specified otherwise.

2.5 Immunohistochemistry

2.5.1 Whole mount antibody staining

Oocytes were freed of their zona pellucida using acidified (pH5.2) Tyrodes medium (Hogan et al, 1986), washed briefly in PBS/PVP (PBS containing 3mg/ml PVP) then fixed in 2.5% PFA for 15 minutes at room temperature. Fixed oocytes were permeabilised in 0.25% Trition x100 in PBS/PVP for 30 minutes then placed in blocking solution (0.1% BSA, 0.01% Tween 20 in PBS) for 15 minutes. Oocytes were incubated in primary antibody solution (blocking solution plus 1° antibody) for 1 hour. The anti Oct-4 (N-19, Santa Cruz; Oct-3, BD Transduction Labs) and anti Stat3 (Santa Cruz) antibodies was used at a 1:100 dilution. Subsequently the oocytes were washed three times with blocking solution for 15 minutes, incubated in an appropriate secondary antibody solution (blocking solution plus appropriate FITC conjugated 2° antibody at 1/100) for 1 hour and rinsed three times in blocking solution prior to mounting in citiflour (Agar Scientific).

2.5.2 Antibody staining of frozen sections

Embryo/tissue were dissected then fixed in 4% PFA for 2 hours at 4°C and washed in PBS (3 x 5 minutes) prior to leaving overnight in 30% sucrose/PBS at 4°C. The embryo/tissue sample was embedded in OCT on dry ice and stored at -80°C. Prior to sectioning the embedded tissue/embryo samples were placed at -20°C for 30 minutes. 10μm sections were cut using a cryostate (Lecia CM1900) and the sections were stored at -20°C. The sections were briefly allowed to air dry before washing quickly with PBS (5 x 2 minutes) at room temperature. The sections were blocked in PBTS (0.1M PBS, 0.1% triton-X 100, 1% BSA) for 2 hours at room temperature, then incubated in a

humidified chamber from 1 hour to overnight with the primary antibody (Cre 1/100; Oct-4 (Oct-3) 1/100; Oct-4 (N-19) 1/100) diluted in PBTS. Subsequently the sections were washed with PBS (3 x 5 minutes) and incubated with the appropriate FITC conjugated secondary antibody (1/100) for 1-2hrs in the dark at room temperature, then washed in PBS (3 x 5 minutes) and mounted in citifluor.

2.6 Imaging

Digital images of whole-mount embryos and sections were acquired using Openlab v.3.0 (Improvison) and a digital camera attached to a Zeiss Stemi SV11 stereoscope with bright field and FITC fluorescence optics. Acquired images were processed using Adobe Photoshop v.7.0 (Adobe Systems Inc.)

Confocal microscopy and imaging was performed by Linda Sharp at the Division of Biomedical and Clinical Sciences within The University of Edinburgh.

Chapter 3: Investigation into achieving targeted inactivation at the floxed Oct-4 locus in germ cells using the TNAP-Cre line.

3.1 Introduction

The embryonic expression pattern of murine Oct-4 suggests that this transcription factor may be a germline determinant in mammals. A proposed model is that Oct-4 is the "master" gene of the germline (Pesce and Scholer, 2000). It would be expected that if this was the case that Oct-4 would be both necessary and sufficient to form the germline from any pluripotent cell in the epiblast and be absolutely required for germ cell identity. Based on this hypothesis it would be expected that deletion of Oct-4 protein in germ cells during their specification and development would result in their loss. To test if Oct-4 is required for germ cell identity a standard gene targeting approach cannot be undertaken because of early embryonic lethality at E3.5 (Nichols *et al*, 1998). One way of circumventing embryonic lethality is to use the Cre-LoxP system of conditional gene inactivation (see section 1.10.1). The Cre expression line TNAP-Cre has previously been shown to cause Cre mediated deletion in PGCs at E9.5 – E10-5 using a Z/AP reporter, such that at E13 approximately 60% of germ cells in the genital ridge had undergone recombination (see section 1.10.2, Figure 1.5 and Lomeli *et al*, 2000). Using this transgenic line in combination with the floxed Oct-4 target gene and a null Oct-4 allele, mice were bred of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype. In these animals it was expected that recombination of the floxed Oct-4 target gene would occur in PGCs and allow investigation into the consequences of loss of Oct-4 in germ cells.

The essential consideration for using the Cre-LoxP system to address a given biological question is the specificity, both spatially and temporally, of excision of the floxed target gene. This is critical where Oct-4 is to be targeted during germ cell development and

specification due to the fact that no reporter gene is activated on recombination of the floxed Oct-4 locus. Any failure of this system could potentially invalidate any results; for instance, in the situation where recombination occurred earlier or later than expected, occurred in a mosaic pattern or did not occur at all. In parallel to investigating spatial and temporal TNAP-Cre mediated deletion mice, were bred of the TNAP-Cre^{+} Oct-4$^{-/loP}$ genotype and investigated for effects on germ cell number. These animals were also crossed to wildtype animals to investigate effects on fertility.

This chapter investigates the spatial and temporal activity of the TNAP-Cre line and the consequence of using this Cre line to inactivate the floxed Oct-4 locus in TNAP-Cre^{+} Oct-4$^{-/loxP}$ animals.

3.2 Experimental strategy

To investigate the activity of the TNAP-Cre line, crosses to reporter lines were performed using floxed target genes that on recombination activated a reporter. The reporter lines used were a silent green fluorescent protein (sGFP) (Gilchrist *et al.* 2003) and the ROSAβgeo26 (R26R) reporter line (Soranio *et al*, 1999). Figure 3.1 shows the genomic structure of the sGFP and R26R reporter lines.

Mouse crosses were performed to generate TNAP-Cre^{+} Oct-4$^{-/loxP}$ embryos and animals. These animals were bred with wildtype animals to investigate their fertility and pups born were genotyped for the status of the floxed Oct-4 allele; recombined or not recombined. To investigate if excision of the floxed Oct-4 locus by TNAP-Cre caused any gross abnormalities in germ cell numbers alkaline phosphatase staining was performed on genital ridges dissected from E12.5 TNAP-Cre^{+} Oct-4$^{-/loxP}$ embryos.

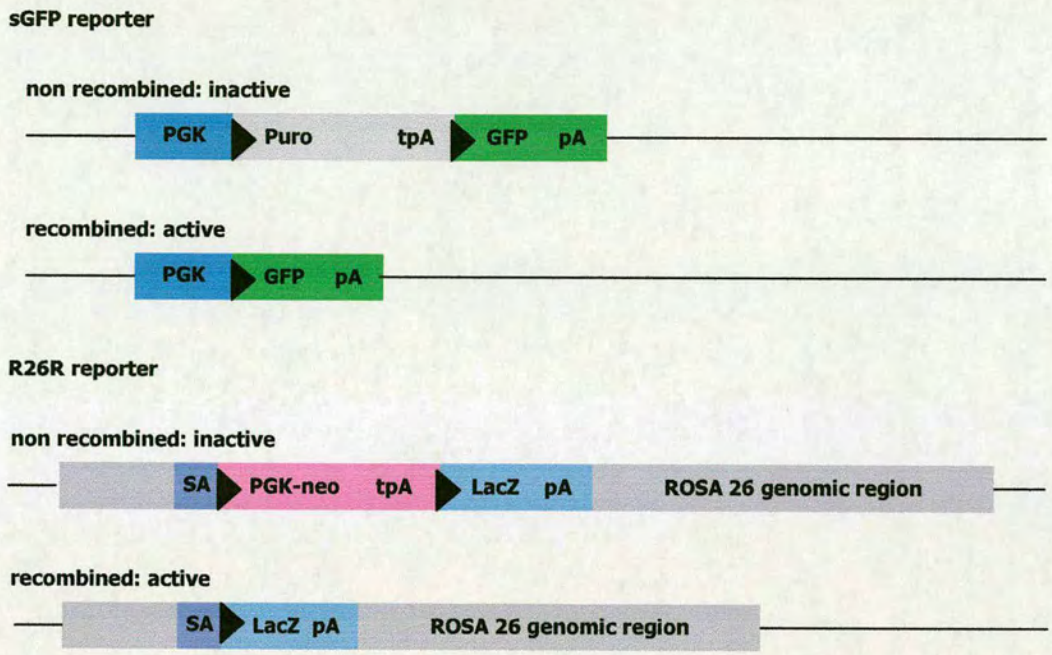


Figure 3.1 Genomic structures of sGFP and R26R reporter lines. The sGFP line was generated by random integration of the indicated construct and consists of a floxed region containing of puromycin selection cassette (Puro pA) flanked by PKG-1 promoter and GFP. The R26R was generated by targeting to the Rosa 26 locus and consists of a PGK neomycin selection cassette (PGK-neo tpA) flanked by the endogenous R26R splice acceptor and a β-galactosidase expression cassette (LacZ pA). In the presence of Cre recombinase the floxed region in each reporter line was excised resulting in the expression of GFP or β-galactosidase.

Figure 3.2 GFP expression in E7.5 embryos obtained from male sGFP x female TNAP-Cre crosses. Panels a, b and c show a double positive TNAP-Cre$^{+/-}$ sGFP$^{+/-}$ embryo. Panel c shows a close up image of the boxed region in panel b. A TNAP$^{-/-}$sGFP$^{+/-}$ embryo is shown in panels d and e. Embryos are shown under U.V and bright field.

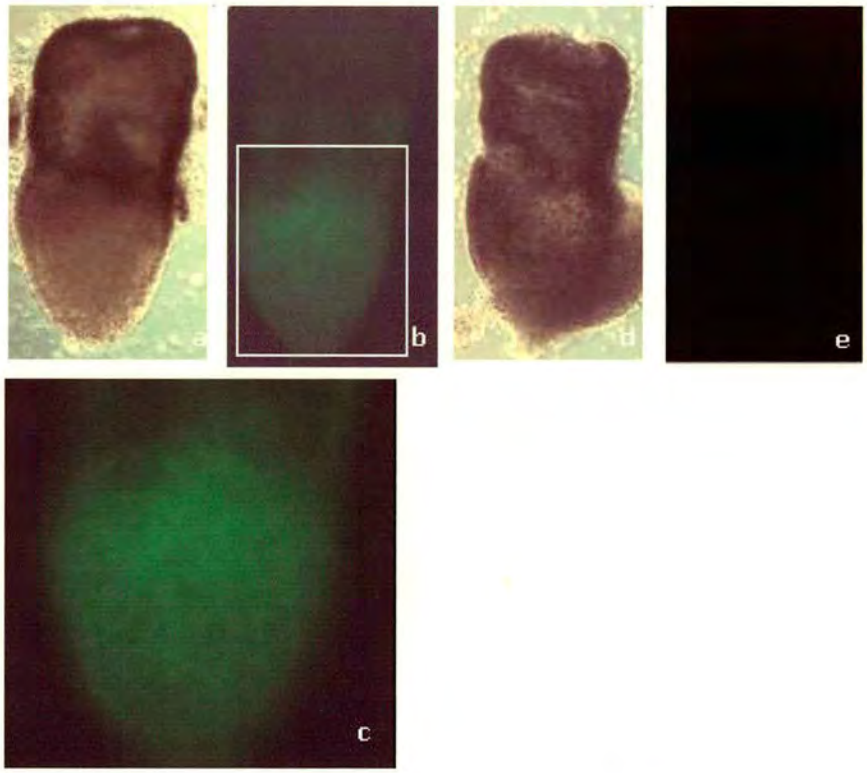


Figure 3.3 GFP expression in E10.5 embryos from male sGFP x female TNAP-Cre crosses. Panels a and b show a double positive TNAP-Cre$^{+/-}$ sGFP$^{+/-}$ embryo. Panels c and d show a TNAP-Cre$^{-/-}$ sGFP$^{+/-}$ embryo. Embryos are shown under U.V and bright field.

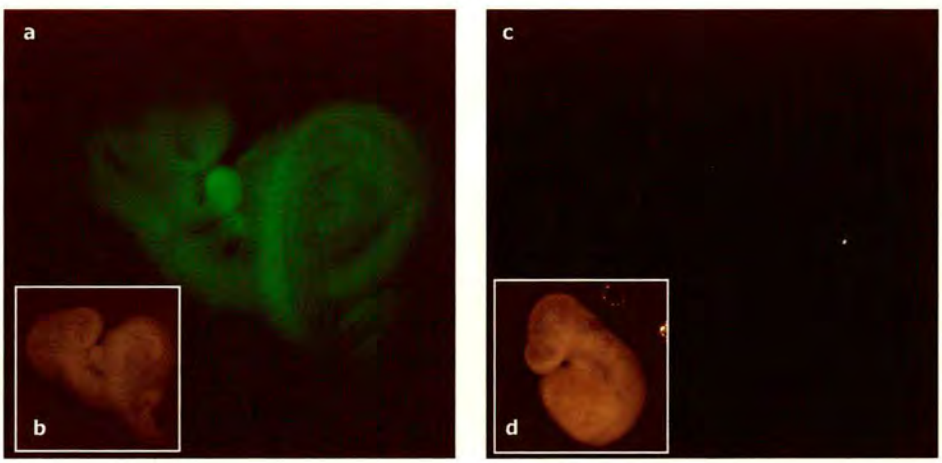
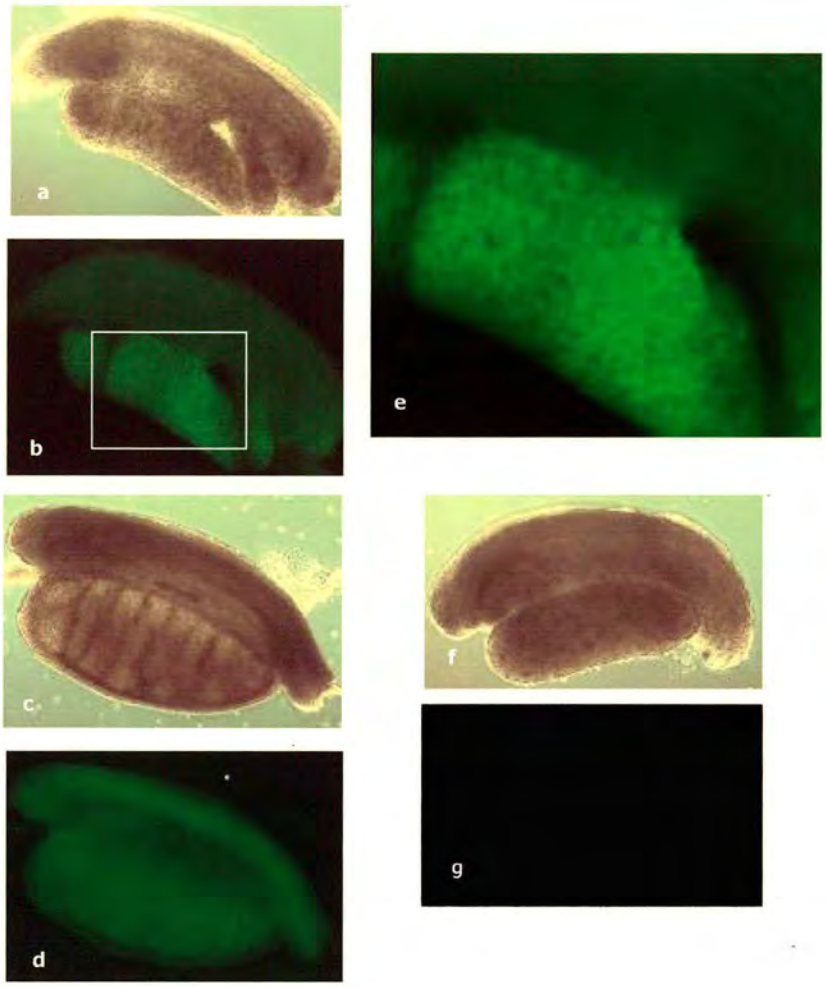


Figure 3.4 GFP expression in genital ridges from E12.5 embryos from male sGFP x female TNAP-Cre crosses. Panels a, b, c, d and e show genital ridges from double positive TNAP-Cre$^{+/-}$ sGFP$^{+/-}$ embryos under light field and U.V. Panel e is a close up image of the boxed region in panel b. Panels f and g show a genital ridge from a wildtype embryo under light field and U.V.



R26R reporter line studies

A second reporter line, R26R, was employed due to the fact that given the intensity of the fluorescent relative to background autofluorescence when using the sGFP reporter lines it was difficult to view any finer details of sGFP activation in the TNAP-Cre$^{+/-}$ sGFP$^{+/-}$ embryos. Mouse crosses were performed between the TNAP-Cre and the R26R lines. Whole E3.5, E7.5, E8.5, E10.5 and E12.5 embryos obtained, and where possible their yolk sacs, were stained for ß-galactosidase activity and genotyped by PCR (see Tables 3.2 and 3.3). Figure 3.5 shows images of embryos stained for ß-galactosidase activity obtained from male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ crosses. Figure 3.6 shows images of embryo stained for ß-galactosidase activity obtained from male TNAP-Cre$^{+/-}$ x female R26R$^{+/-}$ crosses.

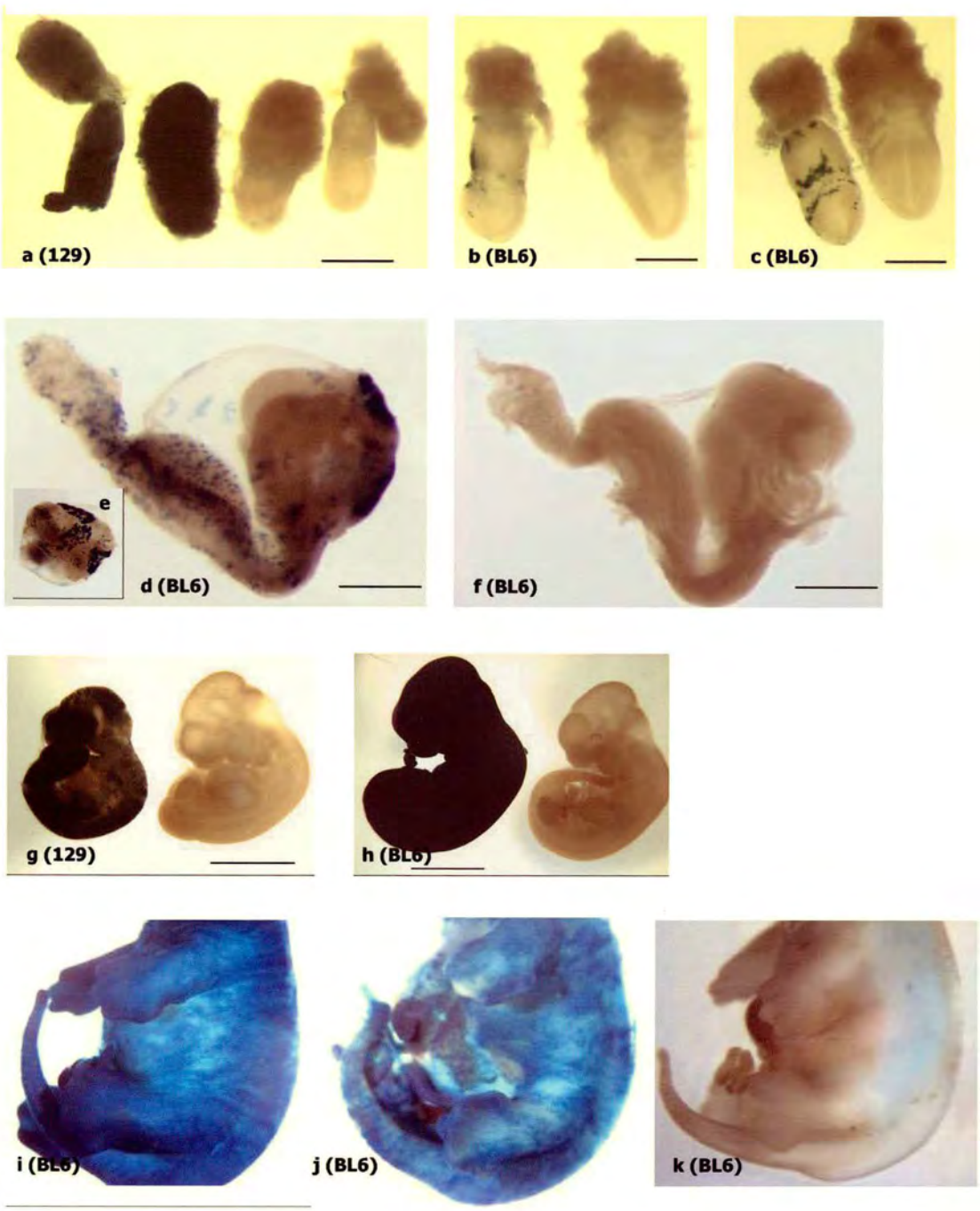
The majority of the TNAP-Cre$^{+/-}$R26R$^{+/-}$ (21/60) stained throughout for β-galactosidase activity, however, some embryos (6/21 TNAP-Cre$^{+/-}$R26R$^{+/-}$ embryos) exhibited a degree of mosacism where β-galactosidase activity was restricted to patches of cells (see Figures 3.5b, c and d and 3.5a). These patches varied in size and position and did not correlate with the expected position of the germ cells. These results are discussed in section 3.4. TNAP-Cre activity was detected ectopically in TNAP-Cre$^{+/-}$ R26R$^{+/-}$ embryos, from E3.5 (see figure 3.6a) onwards and β-galatosidase activity was observed in the embryonic and extraembryonic regions, both throughout and in a mosaic pattern at E7.5 (see Figures 3.5 a, b and c). In later embryos at E8.5, E10.5 and E12.5 ß-galactosidase activity was detected throughout and a few mosaic embryos were observed (see Figures 3.5 and Tables 3.2 and 3.3). Genital ridges stained from TNAP-Cre$^{+/-}$ R26R$^{+/-}$ E12.5 embryos did not show any difference in the level of staining than the rest of the embryo (see Figure 3.6c). Also staining was observed in the yolk sac for these embryos (see Figure 3.6d). Crosses were performed using both male and female TNAP-Cre heterozygotes to investigate the possibility of a TNAP-Cre maternal effect.

The data would indicate that there appears to be no apparent difference in TNAP-Cre activity depending on whether the allele was contributed maternally or paternally with this number of embryos. Furthermore, with this limited number of embryos the genetic background of the TNAP-Cre animals, either 129 or C57BL/6, does not appear to affect the phenotypic outcome. Overall a similar level of mosacism was detected for contribution of the *TNAP-Cre* allele paternally or maternally; 4/12 of TNAP-Cre$^{+/-}$ R26R$^{+/-}$ embryos were mosaic where the *TNAP-Cre* allele was contributed maternally and 2/9 of TNAP-Cre$^{+/-}$ R26R$^{+/-}$ embryos were mosaic where the *TNAP-Cre* allele was contributed paternally (see Table 3.2 and 3.3).

E7.5 male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ (129)		
number	genotype	phenotype
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryonic and extraembryonic regions (see Figure 3.4a)
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryonic and extraembryonic regions (see Figure 3.4a)
3	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4a)
4	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4a)
E7.5 male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ (BL6)		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	patches of stained cells located on anterior side of embryo (see Figure 3.4b and c)
2	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4b and c)
3	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
4	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
E8.5 male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ (129)		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	mosaic patches of stained cells throughout embryo and yolk sac(see Figure 3.4d e)
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	mosaic patches of stained cells throughout embryo and yolk sac
3	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4f)
4	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
5	TNAP-Cre^{-}/R26R$^{+/-}$	no staining observed
E10.5 male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ (129)		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	staining throughout embryo (see Figure 3.4g)
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	staining throughout embryo
3	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4g)
4	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
E10.5 male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ (BL6)		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo (see Figure 3.4h)
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo
3	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4h)
4	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
5	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
E12.5 male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ (129)		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo (see Figure 3.4i)
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo in a mosaic manner (see Figure 3.4j)
3	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo
4	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.4k)
5	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
6	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
7	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
8	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed

Table 3.2 Phenotypes and genotypes observed on analysis of embryos at E7.5, E10.5 and E12.5 from male R26R$^{+/+}$ x female TNAP-Cre$^{+/-}$ crosses. The TNAP-Cre lines were N3 backcrosses to 129 and C57BL/6 (BL6). The R26R line was on a C57BL/6 background.

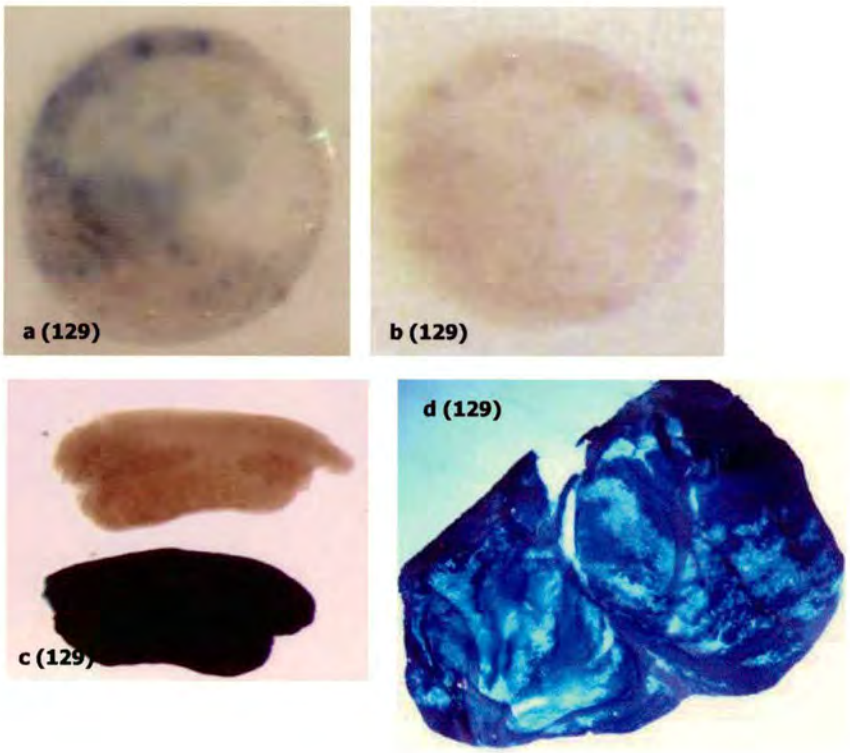
Figure 3.5 β-galactosidase staining of E7.5 (a,b and c), E8.5 (d, e and f), E10.5 (g and h) and E12.5 (i, j and k) embryos obtained from male $R26R^{+/+}$ x female TNAP-$Cre^{+/-}$ crosses. The genetic background of the TNAP-Cre line is indicated. BL6 refers to a C57BL/6 background.



E3.5 male TNAP-Cre$^{+/-}$ (129) x female R26R$^{+/-}$		
number	genotype	phenotype
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	cells of half the blastocyst stained (see Figure 3.5a)
2	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.5b)
3	TNAP-Cre$^{+/-}$/R26R$^{-/-}$	no staining observed
4	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
E7.5 male TNAP-Cre$^{+/-}$ (129) x female R26R$^{+/-}$		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryonic and extraembryonic regions
2	TNAP-Cre$^{+/-}$/R26R$^{-/-}$	no staining observed
3	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
4	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
E7.5 male TNAP-Cre$^{+/-}$ (BL6) x female R26R$^{+/-}$		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryonic and extraembryonic regions
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryonic and extraembryonic regions, appeared mosaic
3	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
4	TNAP-Cre$^{+/-}$/R26R$^{-/-}$	no staining observed
5	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
E10.5 male TNAP-Cre$^{+/-}$ (129) x female R26R$^{+/-}$		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	staining throughout embryo
2	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
3	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
4	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
E10.5 male TNAP-Cre$^{+/-}$ (BL6) x female R26R$^{+/-}$		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo
2	TNAP-Cre$^{+/-}$/R26R$^{-/-}$	no staining observed
3	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed
4	TNAP-Cre$^{+/-}$/R26R$^{-/-}$	no staining observed
5	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
E12.5 male TNAP-Cre$^{+/-}$ (129) x female R26R$^{+/-}$		
1	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo (see Figure 3.5c)
2	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo
3	TNAP-Cre$^{+/-}$/R26R$^{+/-}$	stained throughout embryo
4	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
5	TNAP-Cre$^{-/-}$/R26R$^{-/-}$	no staining observed
6	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed (see Figure 3.5c)
7	TNAP-Cre$^{+/-}$/R26R$^{-/-}$	no staining observed
8	TNAP-Cre$^{-/-}$/R26R$^{+/-}$	no staining observed

Table 3.3 Phenotypes and genotypes observed on analysis of embryos at E7.5, E10.5 and E12.5 from male TNAP-Cre$^{+/-}$ x female R26R$^{+/+}$ crosses. The TNAP-Cre lines were N3 backcrosses to 129 and C57BL/6 (BL6). The R26R line was on a C57BL/6 background.

Figure 3.6 β-galactosidase staining of E3.5 (a and b) genital ridges (c) and yolk sac (d) from E12.5 embryos obtained from male TNAP-Cre$^{+/-}$ x female R26R$^{+/-}$ crosses. The TNAP-Cre lines were N3 backcrosses to 129 and C57BL/6 (BL6). The R26R line was on a C57BL/6 background



3.3.2 *Targeted inactivation of the floxed Oct-4 allele using TNAP-Cre*

Mice/embryos of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype were generated by the following crosses, as shown in Figure 3.7.

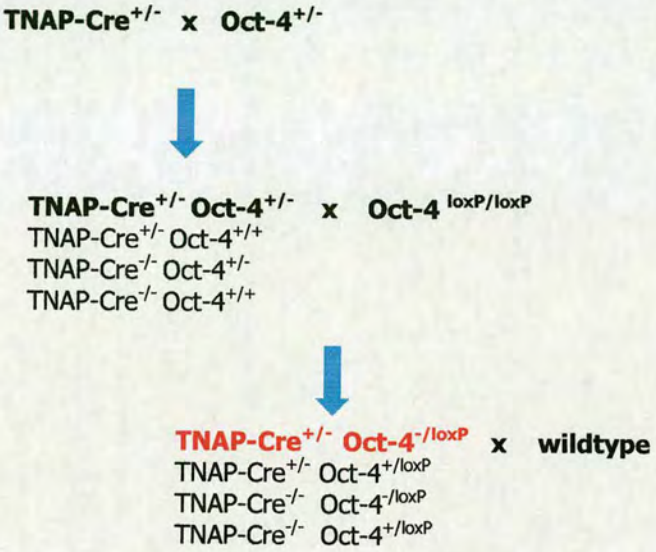


Figure 3.7 Breeding scheme to generate TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice (denoted in red). The four genotypes from TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ x Oct-4 $^{loxP/loxP}$ cross are indicated and theoretically would be expected to be obtained in a 1:1:1:1 Mendelian ratio.

Investigation into a potential negative maternal effect of TNAP-Cre

Given that ectopic TNAP-Cre recombinase mediated deletion occurred throughout the embryo at the sGFP and R26R reporter loci this could also be anticipated at the floxed Oct-4 locus. Work in progress by J. Nichols in Austin Smith's group using a T-Cre line that was active in the epiblast to inactivate the floxed Oct-4 locus has shown that Oct-4 in the epiblast is required for normal development. Due to this potential overlap in expression patterns between the TNAP-Cre and T-Cre lines crosses were performed between TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ and Oct-4 $^{loxP/loxP}$ animals, to firstly identify if the

appropriate mutant embryos were obtained, and secondly if they were obtained in the expected numbers based on the chi-squared calculation where $\chi^2 = \Sigma$ ((observed – expected)^2/expected). Furthermore, χ^2 values were also calculated to ascertain if the maternal or paternal contribution of the *TNAP-Cre* allele caused any deviation from the expected Mendelian ratio of 1:1:1:1.

On analysis of the genotypes of 112 embryos from 16 litters a χ^2 value of 2.5 was calculated (Figure 3.8, Table 3.4). With 3 degrees of freedom, at $p \leq 0.05$, χ^2 value = 7.82. Therefore the calculated χ^2 value suggests the data is in agreement with a Mendelian ratio of 1:1:1:1. A χ^2 value of 1.80 was calculated for 77 embryos from 11 litters where the *TNAP-Cre* allele was contributed paternally (Figure 3.9, Table 3.5). Therefore the calculated χ^2 value suggests the data is also in agreement with a Mendelian ratio of 1:1:1:1. However, a χ^2 value of 11.29 was calculated for 35 embryos from 7 litters where the *TNAP-Cre* allele was contributed maternally (Figure 3.10, Table 3.6). Thus the genotypic ratio is significantly different from the expected Mendelian outcome. Furthermore, if resorbtions are assumed to represent embryos of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype and the χ^2 values recalculated all χ^2 values are nonstatistically significant and are in agreement with the predicted Mendelian ratio of 1:1:1:1. The χ^2 values calculated were as follows; $\chi^2 = 3.00$ for all embryos, $\chi^2 = 3.50$ where the *TNAP-Cre* allele was contributed paternally and $\chi^2 = 4.35$ where the *TNAP-Cre* allele was contributed maternally.

These data may suggest that some of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos where the *TNAP-Cre* allele was contributed maternally were lost during embryogenesis.

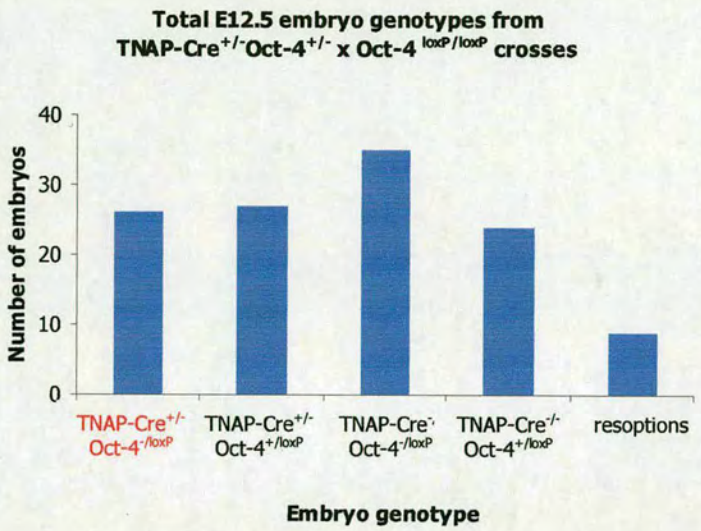


Figure 3.8 Distribution of embryo genotypes from total TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ x Oct-4 $^{loxP/loxP}$ crosses. TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos are indicated in red. 9 resoptions were also recorded.

Genotype	observed number	expected number	(observed – expected)^2/expected
TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$	26	28	0.14
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	27	28	0.04
TNAP-Cre$^{-/-}$Oct-4$^{-/loxP}$	35	28	1.75
TNAP-Cre$^{-/-}$Oct-4$^{+/loxP}$	24	28	0.57
	total = 112		**χ^2 = 2.5**

Table 3.4 Calculation of χ^2 value for total embryo genotypes from the TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ x Oct-4 $^{loxP/loxP}$ cross where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

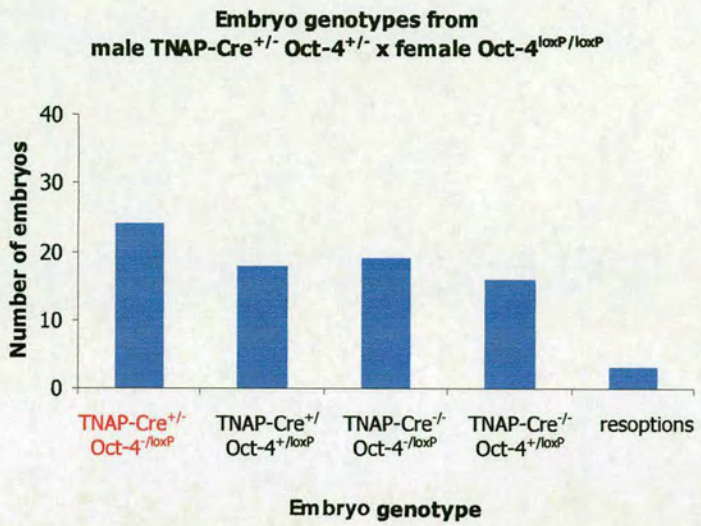


Figure 3.9 Distribution of embryo genotypes from male TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ x female Oct-4 $^{loxP/loxP}$ crosses. 3 resoptions were also recorded.

Genotype	observed number	expected number	(observed – expected)^2/expected
TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$	24	19.25	1.17
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	18	19.25	0.08
TNAP-Cre$^{-/-}$Oct-4$^{-/loxP}$	19	19.25	0.00
TNAP-Cre$^{-/-}$Oct-4$^{+/loxP}$	16	19.25	0.55
	total = 77		**χ^2 = 1.80**

Table 3.5 Calculation of χ^2 value for embryo genotypes from the male TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ x female Oct-4 $^{loxP/loxP}$ cross where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

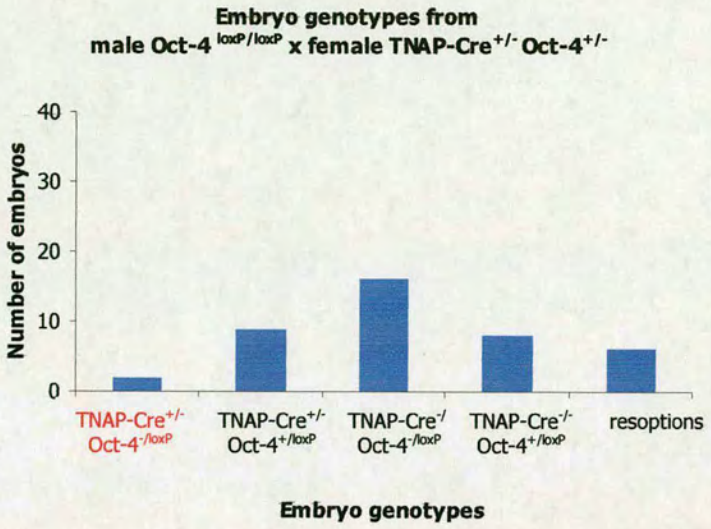


Figure 3.10 Distribution of embryo genotypes from male Oct-4 $^{loxP/loxP}$ x female TNAP-Cre$^{+/-}$ Oct$^{+/-}$ crosses. 6 resoptions were also recorded.

Genotype	observed number	expected number	(observed – expected)^2/expected
TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$	2	8.75	5.21
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	9	8.75	0.01
TNAP-Cre$^{-/-}$Oct-4$^{-/loxP}$	16	8.75	6.01
TNAP-Cre$^{-/-}$Oct-4$^{+/loxP}$	8	8.75	0.06
	total = 35		**χ^2 = 11.29**

Table 3.6 Calculation of χ^2 value for embryo genotypes from the male Oct-4 $^{loxP/loxP}$ x female TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$ where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

Germ cell staining in TNAP-Cre$^{+/-}$ Oct-4$^{/loxP}$ embryos

Alkaline phosphatase is expressed by germ cells and therefore can used as a marker to look for gross abnormalities in germ cell numbers within E12.5 genital ridges. Alkaline phosphatase staining was performed on genital ridges dissected from E12.5 embryos from TNAP-Cre$^{+/-}$ Oct-4$^{-/+}$ x Oct-4$^{loxP/loxP}$ crosses. Tissue samples were taken from all embryos for PCR genotyping and this data has been presented in the previous section. No apparent difference in staining was obvious between genital ridges from TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos and the other three embryo genotypes (TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$, TNAP-Cre$^{-/-}$ Oct-4$^{-/loxP}$ and TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$), see Figure 3.11. This would suggest that mice of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype had no severe reduction in germ cell numbers.

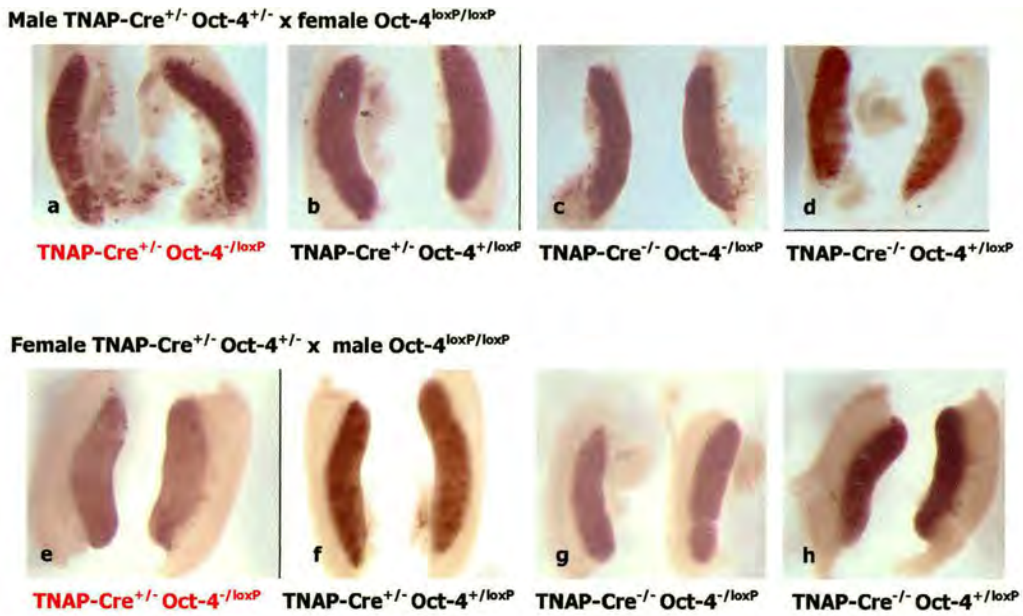


Figure 3.11 Alkaline phosphatase staining of E12.5 genital ridges from embryos from TNAP-Cre$^{+/-}$ Oct-4$^{-/+}$ x Oct-4$^{loxP/loxP}$ crosses. Embryo genotypes are indicated; a-d are genital ridges from male TNAP-Cre$^{+/-}$ Oct-4$^{-/+}$ x female Oct-4$^{loxP/loxP}$ cross and e-h are genital ridges from female TNAP-Cre$^{+/-}$ Oct-4$^{-/+}$ x male Oct-4$^{loxP/loxP}$ cross.

Investigation into the inheritance pattern of the floxed Oct-4 allele

Adult male and female TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice were crossed with wildtype animals to investigate if there were any affects on fertility and to analyse the status of the floxed Oct-4 allele in any pups born. Eight breeding pairs were set up (five male TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ and five female TNAP-Cre$^{+/-}$Oct-4$^{-/loxP}$ animals) and all produced litters, the average litter size being 8. 121 pups born were genotyped by PCR from ear punch samples. On analysis of the genotypes of these 121 pups a χ^2 value of 2.81 was calculated (Table 3.7). With 3 degrees of freedom, at $p \leq 0.05$, χ^2 value = 7.82. A χ^2 value of 4.25 was calculated for 59 pups where the *TNAP-Cre* allele was contributed paternally (Table 3.8). A χ^2 value of 5.76 was calculated for 62 pups where the *TNAP-Cre* allele was contributed maternally (see Table 3.9). None of these values were statistically significant and are in agreement with obtaining the expected genotypes in a 1:1:1:1 Mendelian ratio.

Table 3.10 summarises the overall genotype results and includes the recombination status of the floxed Oct-4 allele; non-recombined, fully recombined or mosaic recombination. Tables 3.11 and 3.12 show the pup genotype results depending whether the *TNAP-Cre* allele was contributed maternally or paternally. The data suggest that in pups that have inherited the floxed *Oct-4* allele recombination only occurs where *TNAP-Cre* is also inherited. If recombination did occur it was normally in a mosaic pattern. However, two pups were obtained where the *TNAP-Cre* allele was contributed maternally in which the *Oct-4* floxed allele had undergone complete recombination. Figure 3.12 shows typical genotyping data obtained from pups born from TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ animals.

Genotype	observed number	expected number	(observed – expected)^2/expected
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	26	30.5	0.60
TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$	32	30.5	0.10
TNAP-Cre$^{+/-}$Oct-4$^{+/-}$	26	30.5	0.60
TNAP-Cre$^{-/-}$Oct-4$^{+/-}$	37	30.5	1.51
	total = 126		**χ^2 = 2.88**

Table 3.7 Calculation of χ^2 value for pup genotypes from total TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x wt crosses where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

Genotype	observed number	expected number	(observed – expected)^2/expected
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	12	14.75	0.51
TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$	10	14.75	1.53
TNAP-Cre$^{+/-}$Oct-4$^{+/-}$	17	14.75	0.34
TNAP-Cre$^{-/-}$Oct-4$^{+/-}$	20	14.75	1.87
	total = 59		**χ^2 = 4.25**

Table 3.8 Calculation of χ^2 value for pup genotypes from male TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x female wt crosses where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

Genotype	observed number	expected number	(observed – expected)^2/expected
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	14	15.5	0.15
TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$	22	15.5	2.73
TNAP-Cre$^{+/-}$Oct-4$^{+/-}$	9	15.5	2.73
TNAP-Cre$^{-/-}$Oct-4$^{+/-}$	17	15.5	0.15
	total = 62		**χ^2 = 5.76**

Table 3.9 Calculation of χ^2 value for pup genotypes from female TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x male wt crosses where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

pup genotype	number	recombination status of floxed Oct-4 allele		
		non-recombined	fully recombined	mosaic
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	26	13	2	11
TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$	32	32	0	0
TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$	26	-	-	-
TNAP-Cre$^{-/-}$ Oct-4$^{+/-}$	37	-	-	-

Table 3.10 Total pup genotypes from TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x wt crosses.

pup genotype	number	recombination status of floxed Oct-4 allele		
		non-recombined	fully recombined	mosaic
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	12	7	0	5
TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$	10	10	0	0
TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$	17	-	-	-
TNAP-Cre$^{-/-}$ Oct-4$^{+/-}$	20	-	-	-

Table 3.11 Pup genotypes from male TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x female wt crosses.

pup genotype	number	recombination status of floxed Oct-4 allele		
		non-recombined	fully recombined	mosaic
TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$	14	6	2	6
TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$	22	22	0	0
TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$	9	-	-	-
TNAP-Cre$^{-/-}$ Oct-4$^{+/-}$	17	-	-	-

Table 3.12 Pup genotypes from female TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x male wt crosses.

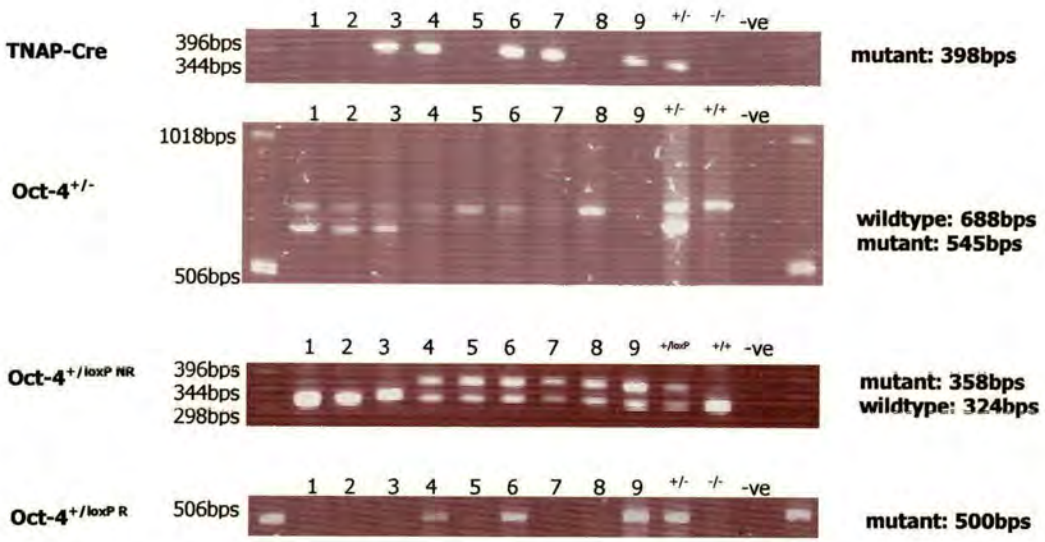


Figure 3.12 Example of typical genotyping data obtained from pups born from TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ animals. The PCR product sizes are indicated for each reaction and the reaction conditions (see Figure 2.2) and primers (see Table 2.1) were as described in the materials and methods in Chapter 2. NR refers to non recombined and R refers to recombined at the floxed Oct-4 locus.

Pup number	ZP3-Cre	Oct-4 null	Oct-4 loxP non recombined	Oct-4 loxP recombined	Pup genotype
1	No	Yes	No	No	TNAP-Cre$^{-/-}$ Oct-4$^{+/-}$
2	No	Yes	No	No	TNAP-Cre$^{-/-}$ Oct-4$^{+/-}$
3	Yes	Yes	No	No	TNAP-Cre$^{+/-}$ Oct-4$^{+/-}$
4	Yes	No	Yes	Yes	TNAP-Cre$^{+/-}$ Oct-4 $^{+/loxPR}$
5	No	No	Yes	No	TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$
6	Yes	No	Yes	Yes	TNAP-Cre$^{+/-}$ Oct-4$^{+/loxPR}$
7	Yes	No	Yes	No	TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$
8	No	No	Yes	No	TNAP-Cre$^{-/-}$ Oct-4$^{+/loxP}$
9	Yes	No	Yes	Yes	TNAP-Cre$^{+/-}$ Oct-4$^{+/loxPR}$

Table 3.13 Summary of genotype data from Figure 3.12

3.3.3 RT-PCR analysis of genital ridges from TNAP-Cre$^{+/-}$ embryos for the presence of Cre recombinase

A Cre recombinase RT-PCR was performed on E12.5 genital ridges from TNAP-Cre$^{+/-}$ embryos to identify whether or not Cre recombinase mRNA was present. No Cre recombinase RNA was detected, see Figure 3.13 below. The β-actin control shows that RNA and cDNA was successfully isolated and generated for all samples, for the Cre recombinase RT-PCR an amplified product was only detected in the positive ES cell control treated with reverse transcriptase (RT).

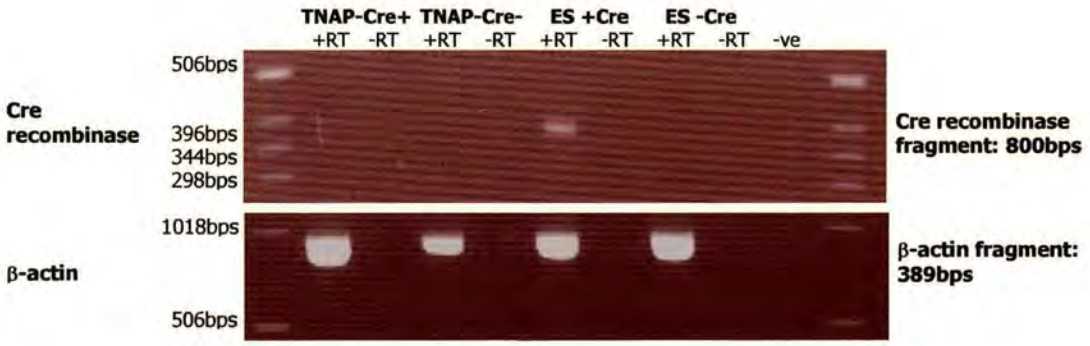


Figure 3.13 Cre recombinase RT-PCR on E12.5 genital ridges from TNAP-Cre$^{+/-}$ embryos. For the embryo samples genital ridges were pooled from four positive and four negative TNAP-Cre$^{+/-}$ and TNAP-Cre$^{-/-}$ embryos respectively. Samples were incubated with and without reverse transcriptase (RT). Positive and negative controls were obtained from an ES cell line that expressed Cre recombinase and wildtype ES cells respectively. –ve refers to a dH_2O only control.

3.4 Discussion

The results from both of the reporter line studies indicated that TNAP-Cre mediated deletion was not confined to germ cells as would be expected based on earlier publications (Lomeli *et al.* 2000; McGregor *et al.* 1995). Instead TNAP-Cre mediated deletion occurred throughout embryo and extraembryonic regions and was detectable in an E3.5 blastocyst. This data would suggest that ectopic TNAP-Cre expression first causes Cre mediated recombination of the reporter loci during early embryogenesis. Once activated in a given cell the reporter loci continually express either GFP from the sGFP reporter or β-galactosidase from the R26R reporter. The earlier in embryogenesis excision occurs the more cell lineages would be expressing GFP or β-galactosidase throughout.

Analysis of genital ridges from E12.5 TNAP-Cre$^{+/-}$ sGFP$^{+/-}$ embryos where the TNAP-Cre allele was contribute maternally did shown that in 2/4 double positive embryos there appeared to an elevation of fluorescence in cells within the genital ridge. This could be explain by the possibility that the early TNAP-Cre mediated deletion occurring in the early embryo was mosaic but gave the appearance of fluorescence throughout the embryo later in development. In the embryos that showed an elevation in levels of fluorescence in cells within the genital ridge the germ cells may have undergone TNAP-Cre mediated recombination at the expected time. The elevated level of fluorescence may be due to more cells in a given region, i.e. the genital ridges, expressing GFP as a result of recombination occurring in a less mosaic manner than earlier in development. This may indicate that in these two particular embryos Cre was expressed in the genital ridges. However, due to the fact that 2 other embryos in the litter of the same genotype did not show this apparent elevation of fluorescence in the genital ridges indicates that the expression pattern of TNAP-Cre was not consistent.

Mosaic TNAP-Cre mediated deletion also occurred in a number of cases and the pattern did not reflect that which was expected for developing/migrating germ cells. No difference was apparent on either the genetic background or whether the *TNAP-Cre* allele was contributed maternally or paternally. This may be due to the small number of embryos used in this study. Furthermore, the presence of embryos which exhibited mosaic TNAP-Cre mediated deletion could not be attributed to particular male/female TNAP-Cre$^{+/-}$ parental animals. The presence of mosaic and non mosaic embryos in the same litter suggest stochastic differences in TNAP-Cre activity between embryos in the same litter that are independent of the original parental allele.

Male and female mice of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype were generated successfully. Analysis of embryos generated from the TNAP-Cre$^{+/-}$ Oct-4$^{-/+}$ x Oct-4$^{loxP/loxP}$ cross suggested that there was a significant loss of embryos of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype where the TNAP-Cre allele was contributed maternally. However a number of adult TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice and embryos were obtained where the *TNAP-Cre* allele was contributed maternally indicating these mice/embryos can be viable. The data from the reporter studies indicates that TNAP-Cre causes Cre mediated deletion at the reporter loci in the ICM and epiblast. If this pattern of excision occurred at the floxed Oct-4 locus is would be expected that the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos would be non viable due to the absolute requirement for Oct-4 in the ICM and epiblast for normal development. The results may suggest that TNAP-Cre exhibits a different excision pattern at the Oct-4 locus as compared to the reporter lines. For instance, if TNAP-Cre mediated recombination at the Oct-4 locus occurs at a low level in a mosaic manner such that the majority of TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos are unaffected and can compensate for the loss of a few ICM or epiblast cells. The loss of TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos could be accounted for by TNAP-Cre mediated deletion at the Oct-4 locus occurring in some embryos at a higher frequency. Differences in recombination

frequencies at the floxed Oct-4 locus could be related to stochastic differences in TNAP-Cre activity.

Breeding of TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice with wildtype animals showed that these animals exhibited normal levels of fertility based on their litter sizes. Analysis of the status of the Oct-4 floxed allele (either recombined or non recombined) in pups born showed that if recombination at the floxed locus did occur it occurred in a mosaic pattern in those pups that also contained the TNAP-Cre allele. This would suggest that recombination occurred post fertilisation and was not related to the status of the originating parental germ cell. No pups were obtained that had undergone total recombination at the floxed Oct-4 locus and were negative for TNAP-Cre indicating that no pups could have been derived from an Oct-4 null germ cell. Recombination at the floxed Oct-4 locus was not detected in all TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP}$ pups. This may be due to the nature of the biopsy for genotyping where an ear punch sample was used. It was conceivable that if other tissues of the TNAP-Cre$^{+/-}$ Oct-4$^{+/loxP\ NR}$ pups had been analysed recombination at the floxed Oct-4 locus may have been detected.

Staining of genital ridges from E12.5 TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos for alkaline phosphatase activity showed there were no gross abnormalities in germ cell number. Taken independently there could be three reasons for this; (i) Cre was not expressed in germ cells and no excision of the floxed Oct-4 locus occurred thus the germ cells maintain Oct-4 expression; (ii) Cre is expressed in germ cells at an earlier point in development and non recombined germ cell (i.e. Oct-4$^{-/loxP\ NR}$) were able to repopulate the genital ridges, or (iii) deletion of Oct-4 occurred in germ cells and Oct-4 is not required for germ cell identity. However, considering the data from the reporter studies and the inheritance pattern of the recombined Oct-4 floxed allele this suggested that TNAP-Cre mediated deletion activity is not what would be expected based on previous publications (Lomeli *et al.* 2000; McGregor *et al.* 1995) and may instead occur

very early in embryogenesis at the floxed Oct-4 locus in a variable mosaic manner. To identify if TNAP-Cre mediated deletion was likely to have occurred in germ cells in the genital ridge it was necessary to elucidate if Cre was expressed in germ cells in the genital ridge. Attempts to use an anti-Cre antibody and Cre *in situ* probe were inconclusive. RT-PCR of genital ridges isolated from E12.5 TNAP-Cre$^{+/-}$ embryos did not detect Cre recombinase mRNA. This would suggest that germs cells present in the genital ridges of the TNAP-Cre Oct-4$^{-/loxP}$ animals/embryos had not undergone recombination and remained Oct-4$^{-/loxP\ NR}$ so could still express Oct-4. If Cre recombinase was expressed earlier in the germ cells during there migratory period it was highly likely that the non recombined germ cells (i.e. Oct-4$^{-/loxP\ NR}$) were able to fully repopulate the genital ridges by normal mitotic division. The recombination observed at the floxed Oct-4 locus was likely due to ectopic TNAP-Cre mediated deletion in the early embryo at a low frequency to permit TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos to be viable.

Chapter 4: Investigation into achieving targeted inactivation at the floxed Oct-4 locus in oocytes during oogensis using the ZP3-Cre line.

4.1 Introduction

Detailed analysis of the expression pattern of Oct-4 during oogenesis by Pesce, M and colleagues (1998) showed that Oct-4 was dramatically downregulated as oocytes entered the first meiotic division followed by a burst of Oct-4 expression in the final stages of meiotic prophase I. This had led to speculation that the downregulation of Oct-4, which occurs in both sexes, may represent one of the molecular triggers involved in the commitment to meiosis (Pesce, M *et al.* 1998). The upregulation observed in oocytes as they exit prophase I has been suggested to indicate an involvement of Oct-4 in oocyte growth and/or acquisition of meiotic competence (Pesce *et al.* 1998).

To investigate if Oct-4 is involved in oocyte growth or meiotic competence by inactivating the Oct-4 gene a standard gene targeting approach cannot be undertaken because of early embryonic lethality at E3.5 (Nichols *et al*, 1998). One way of circumventing embryonic lethality is to use the Cre-LoxP system of conditional gene inactivation (see section 1.10.1) to inactivate the Oct-4 gene prior to the burst of Oct-4 expression as the oocyte exits meiotic prophase I. This burst first occurs at 12 -14 dpp in the first cohort of oocytes exiting meiotic arrest and undergoing meiotic maturation; this burst in Oct-4 expression will be repeated in all oocytes exiting meiotic arrest throughout the reproductive life of the mouse. In order to generate null Oct-4 oocytes the Cre-LoxP system employed must cause deletion at the floxed Oct-4 locus before the first burst of Oct-4 expression at 12-14dpp.

The expression pattern of Zona Pellucida 3 (ZP3) expressed by the oocyte during oogenesis has been well characterised (Philpott *et al.* 1987), see Figure 4.1. Analysis of

the developmental expression of ZP3 during oogenesis by *in situ* hybridisation showed that ZP3 mRNA was first detectable in oocytes 20μm in diameter (early growth phase), and significant accumulation occurred as the oocyte grew peaking at 220fg (0.1 – 0.2% of total p(A^+) RNA) in 50μm oocytes. A dramatic decline in expression was detected once the oocytes had reached 60 - 65μm in diameter that coincided with the latter stages of growth and meiotic maturation. Ovulated eggs were found to contain very low levels of ZP3 mRNA; this mRNA represented no more than 0.04% of total mRNA and did not appear to be translated. Northern blot analysis of ZP3 expression in ovaries showed that ZP3 transcripts were first detected in ovaries from 1-2dpp (approximately 10 – 20 μm in diameter) mice, reaching a maximal level in ovaries from 9dpp (approximately 35μm in diameter) mice and by 15dpp (approximately 50μm in diameter) has decreased to 50% of the maximal levels. Further work using the upstream region of the ZP3 gene to drive a luciferase reporter construct showed that luciferase activity increased substantially during the first 21 days after birth, peaking at approximately 18dpp (Lira *et al.* 1990).

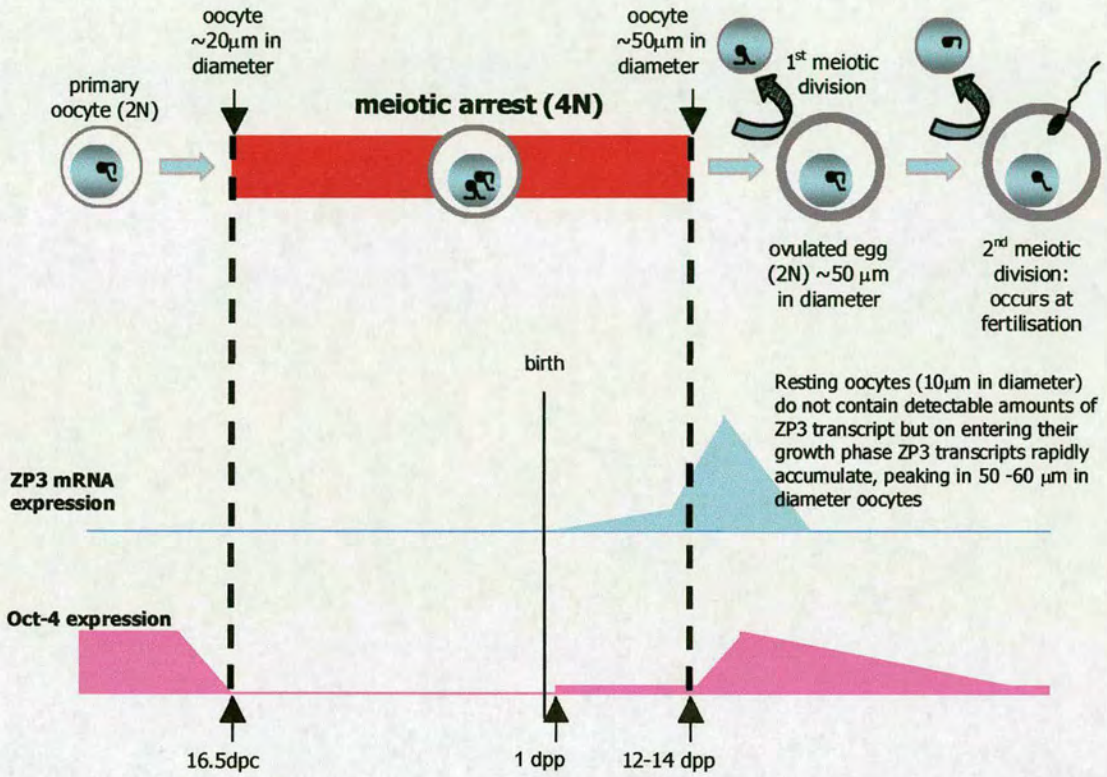


Figure 4.1 Schematic illustration of the timing of Oct-4 expression in relation to ZP3 mRNA expression.

The Cre expression line ZP3-Cre has previously been shown to be specific to growing oocytes from the early stages of oogenesis and cause recombination of a target floxed gene at a very high efficiency of 100% (Lewandoski *et al.* 1997) (see section 1.10.2). The upstream control region used to drive Cre expression by ZP3 was the same as that used to drive expression of the luciferase reporter as discussed previously. Using ZP3-Cre in combination with the floxed Oct-4 target gene and a null Oct-4 allele mice were bred of the ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype. In these animals it was expected that recombination of the floxed Oct-4 target gene would occur in the diploid primary/early oocyte onwards, thus generating oocytes that were null for Oct-4, and allow investigation into the consequences of loss of Oct-4 during oogenesis. Figure 4.2 summaries the genotypic status at the Oct-4 locus at the chromosomal level as oogenesis proceeds. During oogenesis it is expected that recombination at the floxed Oct-4 locus will occur generating Oct-4 null oocytes.

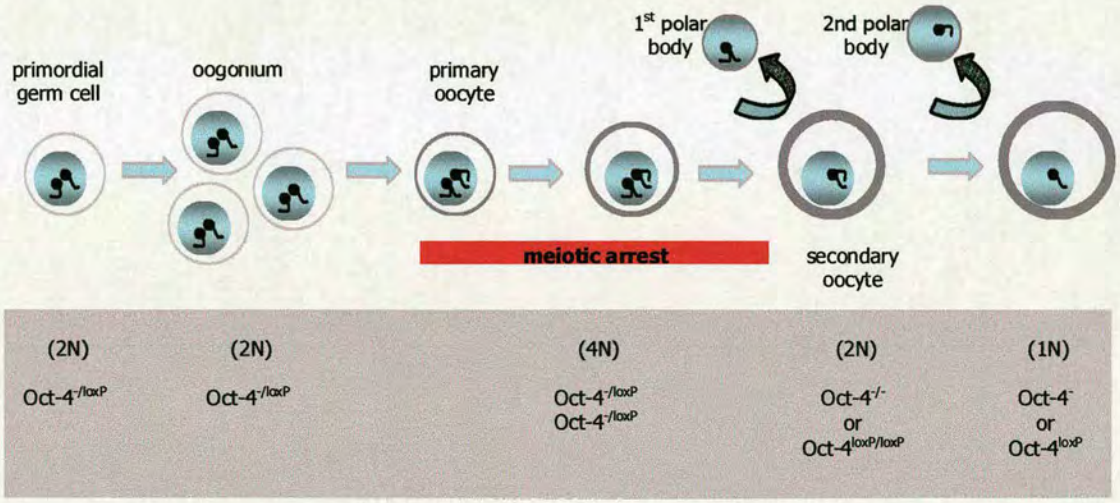


Figure 4.2 Summary of the genotypic status at the Oct-4 locus at the chromosomal level during oogenesis. Extrusion of the 2nd polar body occurs at fertilisation so the oocyte does not exist as a true 1N status, it is indicated here to show that the Oct-4 locus in the oocyte will either be Oct-4^{loxP} or Oct-4^{null}.

Similarly to the previous chapter, where TNAP-Cre was used to direct recombination at the floxed Oct-4 allele, the use of the ZP3-Cre line requires analogous considerations. Since this is a Cre-loxP based approach for achieving spatial and temporal gene inactivation one essential consideration is the specificity of the system. In parallel to investigating the temporal and spatial ZP3-Cre mediated deletion, female mice were bred of the ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype and crossed to wildtype animals to investigate effects on fertility and inheritance pattern of the floxed Oct-4 allele (either recombined or non-recombined) in any pups born.

This chapter investigates the spatial and temporal activity of the ZP3-Cre line and the consequence of using this Cre line to inactivate the floxed Oct-4 locus during oogenesis.

4.2 Experimental strategy

To investigate the activity of the ZP3-Cre line crosses to sGFP and R26R reporter lines were performed. Recombination at these loci resulted in activation of the reporter gene resulting in the expression of GFP or β-galactosidase respectively. The genomic structures are shown in Figure 3.1.

Mouse crosses were performed to generate ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos and animals (see Figure 4.8). These animals were bred with wildtype animals to investigate their fertility and pups born were genotyped for the status of the floxed Oct-4 allele; recombined or not recombined.

4.3 Results

4.3.1 ZP3-Cre reporter studies

sGFP reporter lines studies

Mouse crosses were performed between male ZP3-Cre and female sGFP line to generate double heterozygous female offspring where ZP3-Cre activity was confined to developing oocytes. Oocytes were examined from whole and disaggregated ovaries under U.V light using a benchtop microscope and by confocal microscopy. Figure 4.3 shows whole ovaries from ZP3-Cre$^{+/-}$ sGFP$^{+/-}$ and ZP3-Cre$^{-/-}$ sGFP$^{+/-}$ mice under U.V and bright field. Bright green spherical dots were observed throughout double positive ovaries (see Figure 4.3 a), which were presumed to be the oocytes. However, in both wildtype and mutant ovaries there was a degree of background fluorescence which precluded vision. Vibratome sections were cut of these ovaries but background

autofluorescence precluded vision and also fixing the tissue in 4% paraformaldehyde in order to section it severely affected the levels of fluorescence. This was improved by viewing disaggregated non-fixed ovaries by confocal microscopy and demonstrated that GFP activity was always confined to the oocytes and not the surrounding tissue. Full grown oocytes, approximately 80μm in diameter, were brightly fluorescent throughout whereas less mature oocytes, approximately 40μm in diameter exhibited a lower degree of fluorescence. Figure 4.4 shows confocal images of disaggregated ovaries from ZP3-Cre$^{+/-}$ sGFP$^{+/-}$ and ZP3-Cre$^{-/-}$ sGFP$^{+/-}$ mice.

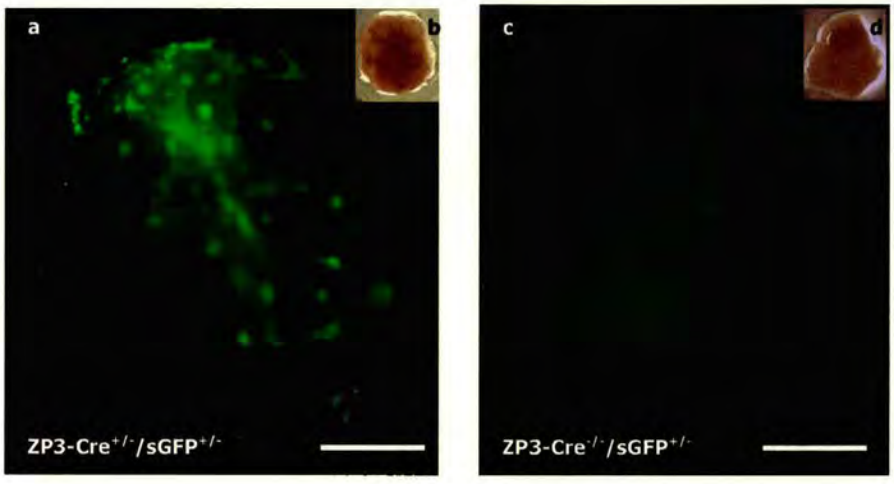


Figure 4.3 Images of whole ovaries from ZP3-Cre$^{+/-}$ sGFP$^{+/-}$ (a and b) and ZP3-Cre$^{-/-}$/sGFP$^{+/-}$ (c and d) six week old female mice under U.V light and bright field. The scale bar represents 0.5mm.

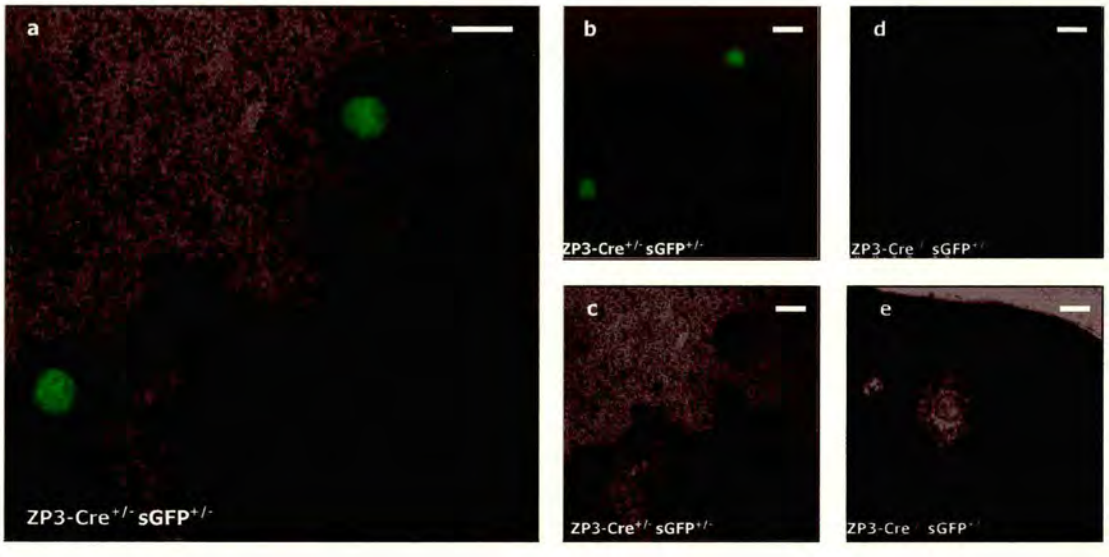


Figure 4.4 Confocal images of disaggregated ovaries from ZP3-Cre$^{+/-}$ sGFP$^{+/-}$ (a, b and c) and ZP3-Cre$^{-/-}$sGFP$^{+/-}$ (d and e) six week old female mice. The scale bar represents 100μm.

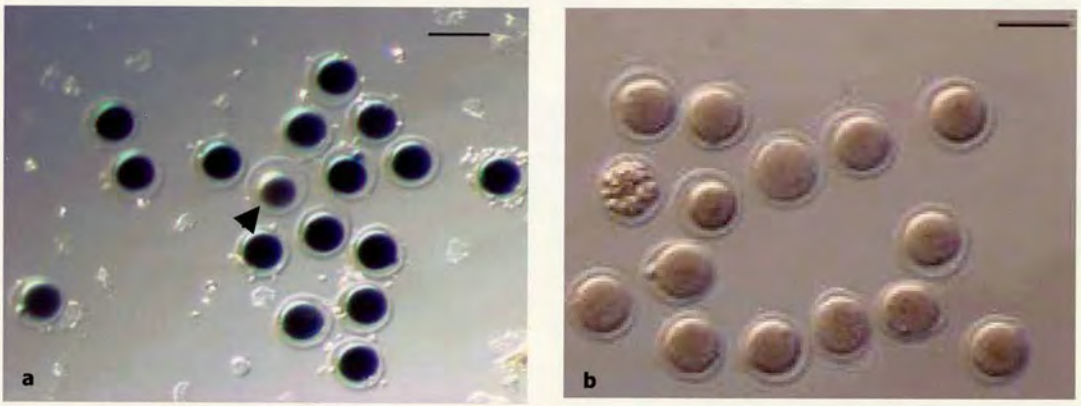


Figure 4.5 β-galactosidase stained superovulated oocytes from ZP3-Cre$^{+/-}$ R26R$^{+/-}$ (a) and ZP3$^{-/-}$ R26R$^{+/-}$ (b) female mice. The oocyte marked with the arrow took a few hours longer to stain than the other oocytes but it did appear slightly abnormal in appearance due to the larger than average zona pellucida.

Mouse genotype	Number of oocytes	β-galactosidase staining
Zp3-Cre$^{+/-}$ R26R$^{+/-}$	17 (1 mouse)	All stained (1 took a few hours longer than others)
ZP3-Cre$^{-/-}$ R26R$^{+/-}$	14 (1 mouse)	none stained
ZP3-Cre$^{+/-}$ R26R$^{-/-}$	15 (1 mouse)	none stained
ZP3-Cre$^{-/-}$ R26R$^{-/-}$	30 (2 mice)	none stained

Table 4.1 Number of β-galactosidase stained superovulated oocytes from ZP3-Cre$^{+/-}$ R26R$^{+/-}$, and ZP3$^{-/-}$ R26R$^{+/-}$, ZP3$^{+/-}$ R26R$^{-/-}$ and wildtype female mice.

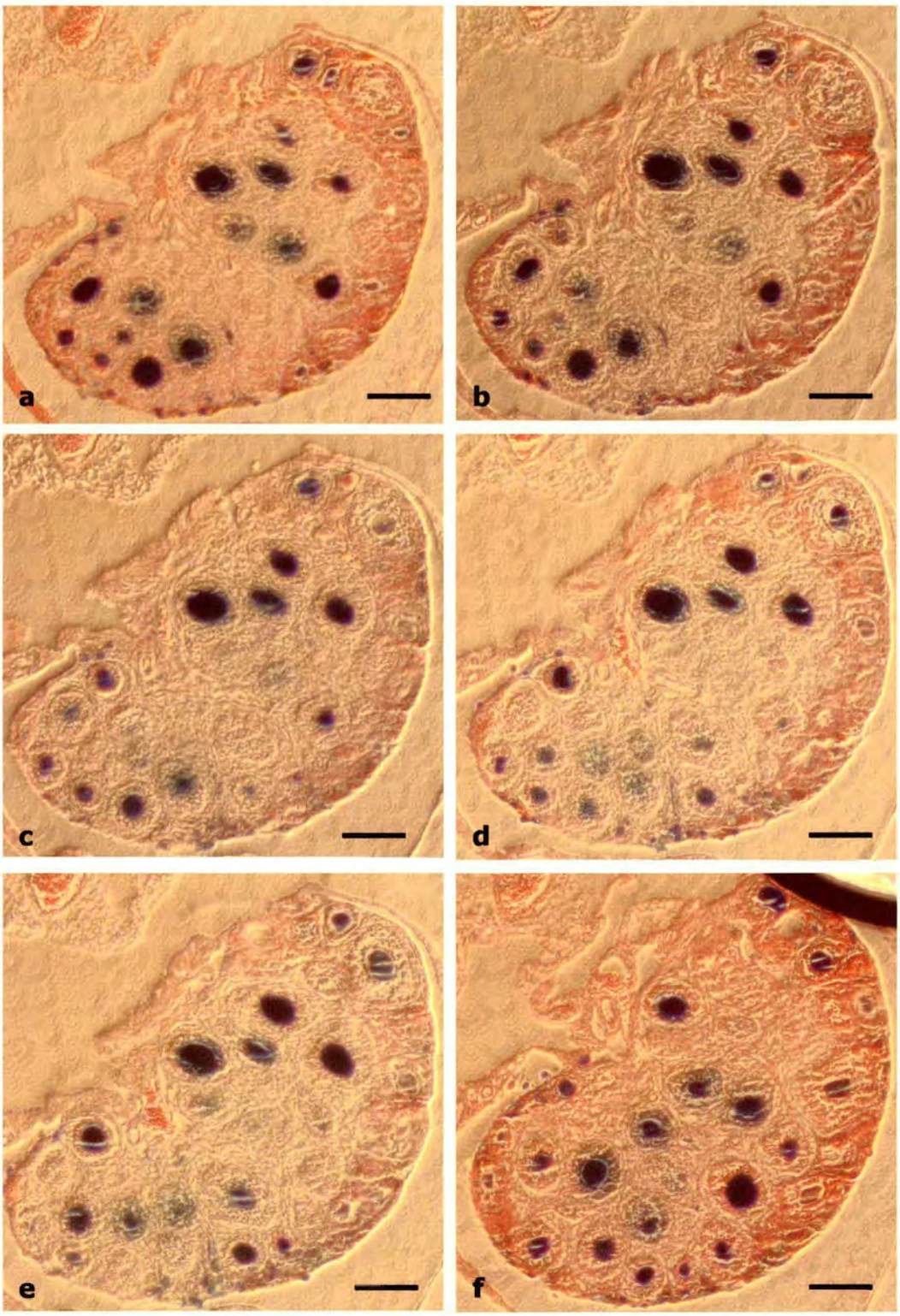


Figure 4.6 7μm serial sections through β-galactosidase stained 21dpp ovary from a ZP3-Cre$^{+/-}$ R26R$^{+/-}$ mouse. Sections were counterstained with eosin. The scale bar represents 100 μm.

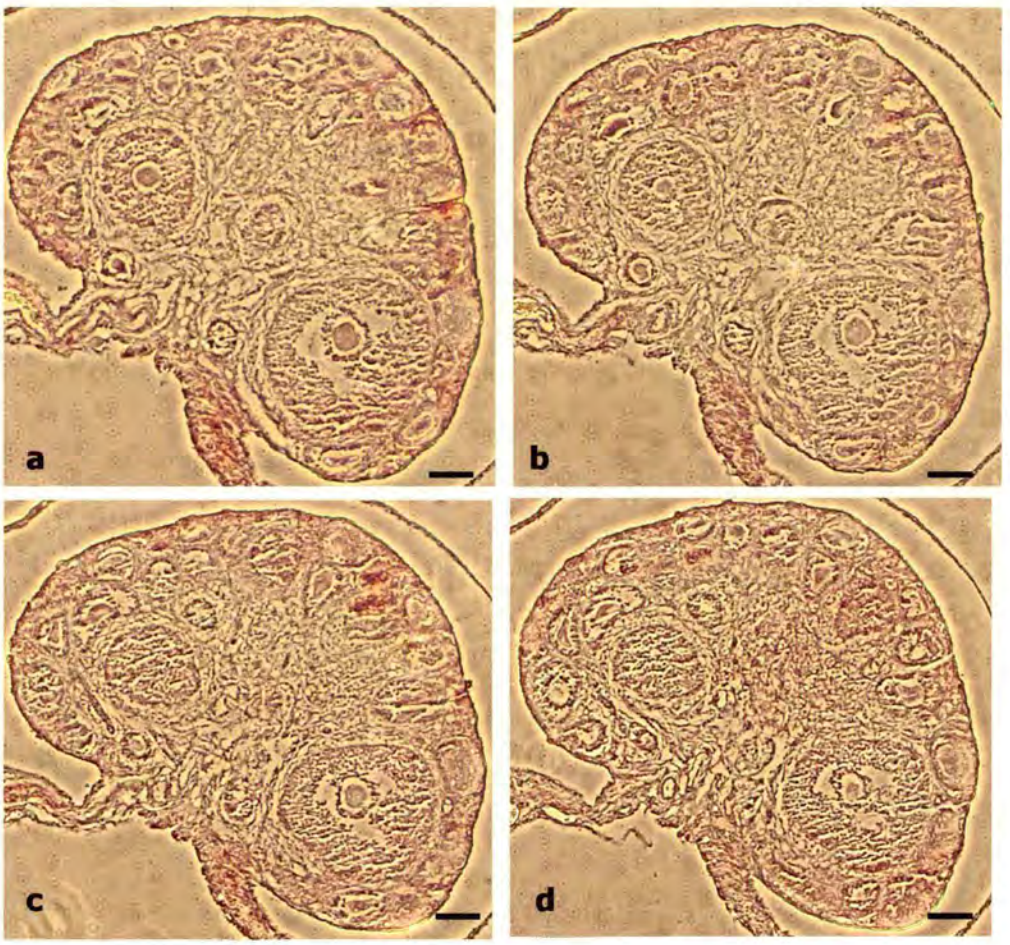


Figure 4.7 7μm serial sections through β-galactosidase stained 21dpp ovary from ZP3-Cre$^{-/-}$ R26R$^{+/-}$ mouse. Sections were counterstained with eosin. The scale bar represents 100 μm.

Mouse genotype	Total number of oocytes (> 20μm) in 10 x 7μm sections	Number of stained oocytes (> 20μm) in 10 x 7μm sections.
ZP3-Cre$^{+/-}$ R26R$^{+/-}$		
1	20	20
2	18	18
3	23	23
4	35	35
5	17	17
ZP3-Cre$^{-/-}$ R26R$^{+/-}$		
1	17	0
2	22	0

Table 4.2 Number of stained individual oocytes in 7μm serial sections from β-galactosidase stained ovaries from five ZP3-Cre$^{+/-}$ R26R$^{+/-}$ and two ZP3-Cre$^{+/-}$ R26R$^{-/-}$ 21dpp female mice.

4.3.2 *Targeted inactivation of the floxed Oct-4 allele using ZP3-Cre*

Female mice of the ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype were produced by the following breeding scheme, as shown in figure 4.8. To generate female mice that will only exhibit ZP3-Cre activity in their ovaries a male ZP3-Cre$^{+/-}$ Oct-4$^{+/-}$ has to be crossed with a female Oct-4 $^{loxP/loxP}$. The reverse cross could have resulted in targeted recombination occurring throughout the pups of a ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ genotype due to ZP3-Cre activity in the oocyte at fertilisation (Lewandoski *et al*, 1996).

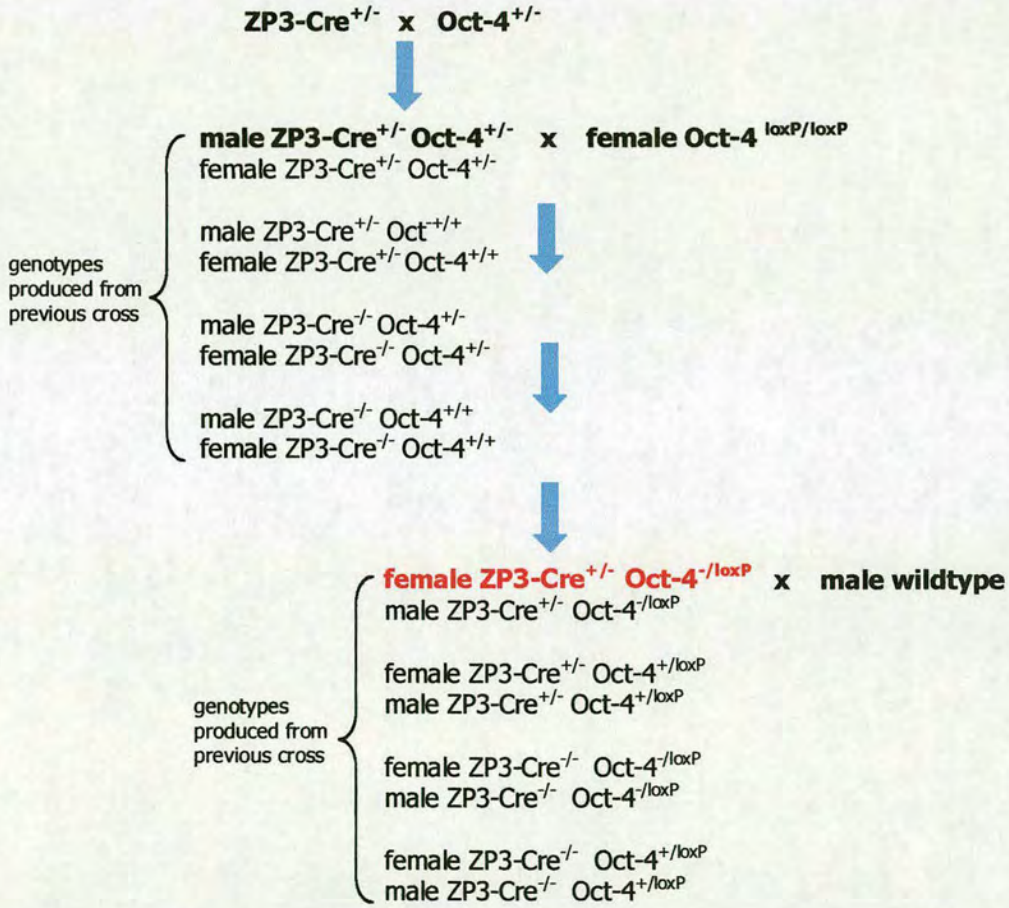


Figure 4.8 Breeding scheme to generate female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice (denoted in red). The four genotypes from male ZP3-Cre$^{+/-}$ Oct-4$^{+/-}$ x female Oct-4 $^{loxP/loxP}$ cross are indicated and theoretically would be expected to be obtained in a 1:1:1:1 ratio. Female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ would be expected to be obtained 1/8.

Investigation into the inheritance pattern of the floxed Oct-4 allele

Adult female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ animals were crossed with wildtype animals to investigate if there were any effects on fertility and to analyse the status of the floxed allele in any pups born.

Female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice were all fertile and 77 pups/embryos were born from 12 females giving an average litter size of 6.4 pups. Pups/embryo genotypes from this cross would be expected to follow a 1:1:1:1 Mendelian ratio of the following genotypes; ZP3-Cre$^{+/-}$ Oct-4$^{-/+}$, ZP3-Cre$^{+/-}$ Oct-4$^{+/-}$, ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$ and ZP3-Cre$^{-/-}$ Oct-4$^{+/loxP}$. On analysis of the genotypes of these 77 pups/embryos a χ^2 value of 5.35 was calculated (Figure 4.9, Table 4.3). With 3 degrees of freedom, at $p \leq 0.05$, χ^2 value = 7.82. Therefore the calculated χ^2 value is in agreement with the expected 1:1:1:1 Mendelian ratio. No effect was observed on litter size with increasing age of the female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice. It may have been expected that if ZP3-Cre caused recombination at the floxed Oct-4 locus in oocytes post the initial burst at 12-14dpp oocytes undergoing maturation later in the reproductive life of the mouse may have been effected.

Furthermore, it would be expected that in these pups there would be a 1:1 ratio between those animals that had inherited the floxed Oct-4 allele and those that had inherited the Oct-4 null allele from the parental female mouse. On analysis of the Oct-4 null or Oct-4 floxed recombined genotypes of the 77 pups/embryos a χ^2 value of 4.68 was calculated, see Table 4.5. With 1 degree of freedom, at $p \leq 0.05$, χ^2 value = 3.84. This value indicates that there is a significant difference (i.e. there is only a 5% chance that the calculated χ^2 value would occur by chance) between the obtained and expected ratio and may suggest the loss of a proportion of oocytes where recombination at the floxed Oct-4 locus has occurred during oogensis. However, this is unlikely to be a true

result because it would be expected that if Oct-4 null oocytes are not viable both Oct-4^{-} and Oct-4 $^{floxed\ R}$ oocytes would be lost simultaneously post recombination at the floxed Oct-4 locus (see Figure 4.2). The numbers obtain may reflect the relatively small number of animals/embryos in this study and the possibility of genotyping errors.

Analysis of the status of the floxed allele showed that in all pups, irrespective of whether or not ZP3-Cre was present, the floxed allele was always recombined (see Table 4.4). No mosacism was observed. Recombination was not observed in 37 pups obtained from female ZP3-Cre$^{-/-}$ Oct-4$^{-/loxP}$ mice when crossed to wildtype males; 18 ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$ and 19 ZP3-Cre$^{-/-}$ Oct-4$^{+/loxP}$. An example of typical genotyping data is shown in Figure 4.10 and Table 4.6.

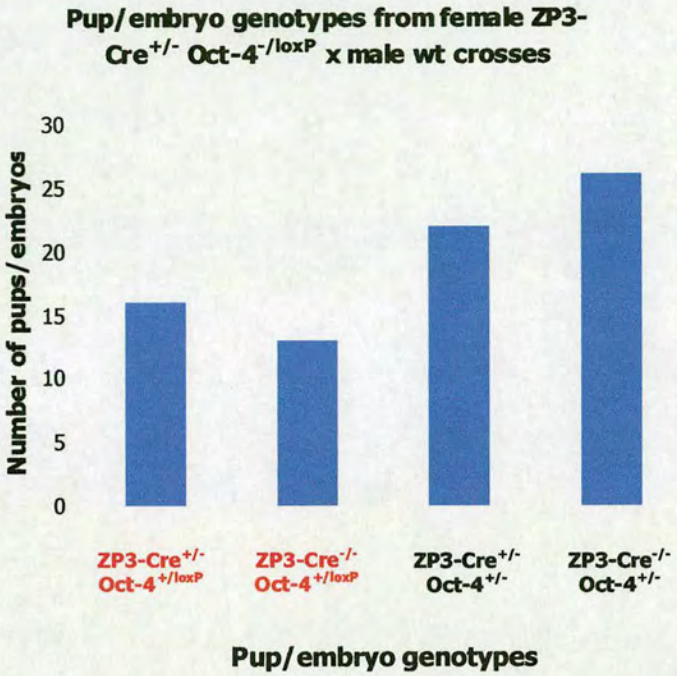


Figure 4.9 Distribution of pup/embryo genotypes from female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x male wt crosses. No resorbtions were recorded for the embryo data.

Genotype	observed number	expected number	(observed – expected)^2/expected
ZP3-Cre$^{+/-}$ Oct-4$^{+/loxP}$	16	19.25	0.55
ZP3-Cre$^{-/-}$ Oct-4$^{+/loxP}$	13	19.25	2.03
ZP3-Cre$^{+/-}$Oct-4$^{+/-}$	22	19.25	0.40
ZP3-Cre$^{-/-}$Oct-4$^{+/-}$	26	19.25	2.37
	total = 77		**χ^2 = 5.27**

Table 4.3 Calculation of χ^2 value for embryo genotypes from the female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x male wt cross where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

Genotype	number	recombination status of floxed Oct-4 allele		
		non-recombined	fully recombined	mosaic
ZP3-Cre$^{+/-}$ Oct-4$^{+/loxP}$	16	0	16	0
ZP3-Cre$^{-/-}$ Oct-4$^{+/loxP}$	13	0	13	0
ZP3-Cre$^{+/-}$Oct-4$^{+/-}$	22	-	-	-
ZP3-Cre$^{-/-}$Oct-4$^{+/-}$	26	-	-	-

Table 4.4 Total pup genotypes from female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x male wt crosses.

Phenotype	observed number	expected number	(observed – expected)^2/expected
Oct-4$^{+/loxP}$	29	38.5	2.34
Oct-4$^{+/-}$	48	38.5	2.34
	total = 77		**χ^2 = 4.68**

Table 4.5 Calculation of χ^2 value for original oocyte genotypes from the female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ x male wt cross where $\chi^2 = \Sigma$ ((observed – expected)^2/expected).

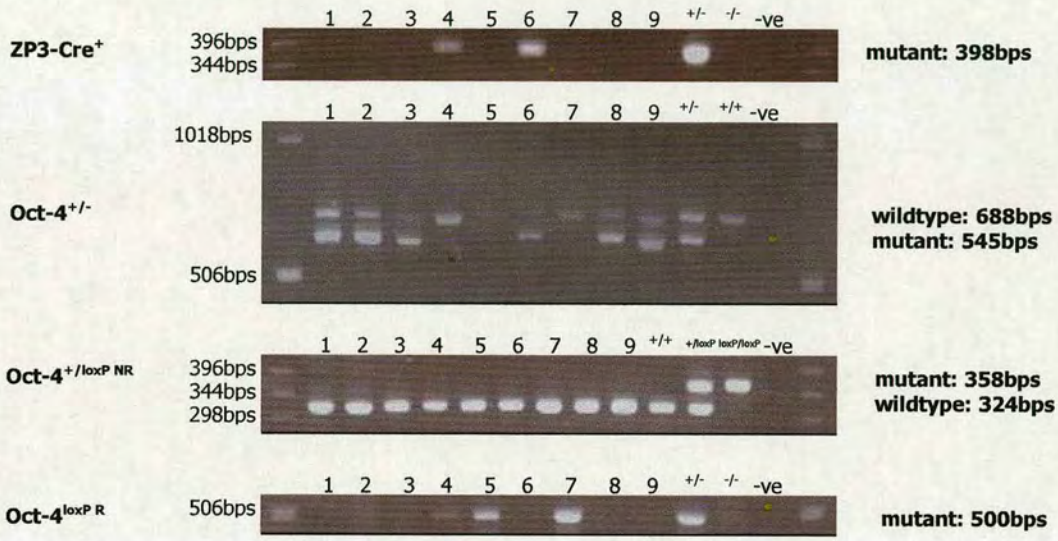


Figure 4.10 Example of typical genotyping data obtained from pups born from ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ females crossed to wt males. The PCR product sizes are indicated for each reaction and the reactions conditions (see Figure 2.2) and primers (see Table 2.1) are as described in the materials and methods in chapter 2. NR refers to non recombined, R refers to recombined. Control DNA was obtained from stock mouse lines.

Pup number	ZP3-Cre	Oct-4 null	Oct-4 loxP non recombined	Oct-4 loxP recombined	Pup genotype
1	No	Yes	No	No	ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$
2	No	Yes	No	No	ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$
3	No	Yes	No	No	ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$
4	Yes	No	No	Yes	ZP3-Cre$^{+/-}$ Oct-4 $^{+/loxPR}$
5	No	No	No	Yes	ZP3-Cre$^{-/-}$ Oct-4$^{+/loxPR}$
6	Yes	Yes	No	No	ZP3-Cre$^{+/-}$ Oct-4$^{+/-}$
7	No	No	No	Yes	ZP3-Cre$^{-/-}$ Oct-4$^{+/loxPR}$
8	No	Yes	No	No	ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$
9	No	Yes	No	No	ZP3-Cre$^{-/-}$ Oct-4$^{+/-}$

Table 4.6 Summary of genotype data from figure 4.10

4.4 Discussion

The results from the reporter line studies indicate that ZP3-Cre mediated deletion occurred in oocytes during oogenesis. Recombination at the sGFP locus was detectable in 40μm oocytes, which corresponded to a time at which the oocyte would be growing during early to mid meiotic arrest. Considerable background autofluorescence prevented detailed investigation into the activation of the sGFP locus by the ZP3-Cre allele so a second reporter was employed; R26R, that on Cre mediated recombination expressed β-galactosidase. Superovulated oocytes from ZP3-Cre$^{+/-}$ R26R$^{+/-}$ female mice all stained positively for β-galactosidase indicating that ZP3-Cre mediated deletion was occurring prior to the point of ovulation at this reporter locus. The time point of ZP3-Cre activity was critical to this study due to the fact that a burst of Oct-4 expression occurs as the oocyte re-enters the meiotic cycle which first occurs at 12-14 dpp in female mice. Analysis of β-galactosidase activity in sectioned ovaries from 21dpp mice showed that R26R reporter activity was present in the majority of oocytes and detectable in oocytes from 20μm in diameter. This size of oocyte corresponded to oocytes in the early growth phase at the beginning of meiotic arrest.

Female ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice were fertile and analysis of the inheritance pattern of the floxed Oct-4 allele (either recombined or not recombined) showed that all pups born that had inherited the Oct-4 floxed allele had undergone recombination. No mosacism was detected indicating that the recombination has occurred in the oocyte prior to fertilisation or in the one cell embryo. The information from the reporter line studies suggested that ZP3-Cre mediated deletion on the reporter loci occurred during the meiotic arrest period. Therefore these results may indicate the Oct-4 is not required during oogenesis. However, due to the fact that no direct information was known about the exact time of ZP3-Cre mediated deletion at the floxed Oct-4 locus and that

Cre mediated deletion can vary between target loci the results from the reporter studies may not necessarily reflect the time of the Cre mediated deletion at the floxed Oct-4 locus. Furthermore, it was not possible to deduce whether or not Oct-4 protein or mRNA were present in oocytes of mutant ovaries due to inconclusive results using anti-Oct-4 antibodies and an Oct-4 *in situ* probe. Analysis of the genotypes of pups born from ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ females showed that pups were obtained of the expected genotypes in a 1:1:1:1 Mendelian ratio but the inheritance of either the floxed recombined or Oct-4 null allele was not in a 1:1 Mendelian ratio possibly suggesting the loss of some Oct-4$^{-/floxed\ recombined}$ oocytes. This cannot be the case since loss of the recombination at the floxed Oct-4 allele would generate Oct-4$^{-/floxed\ recombined}$ oocytes that if not viable would result in their loss, i.e. loss of both Oct-4^{-} and Oct-4$^{floxed\ recombined}$ alleles, see Figure 4.2.

Chapter 5: Discussion

Embryonic expression data of the pattern of murine Oct-4 suggests this transcription factor may be a germline determinant. A hypothesis has been proposed where Oct-4 is the "master" gene of the germline (Pesce and Scholer, 2000). Based on this hypothesis it would be expected that Oct-4 would be necessary to form the germline from any of the pluripotent cells in the epiblast and be absolutely required for germ cell identity. As such, deletion of Oct-4 protein in germ cells during their specification and development would result in their loss by death or possibly somatic differentiation. Furthermore, Oct-4 is also expressed during oogenesis. Expression is dramatically downregulated when oocytes are meiotically arrested during prophase I of the first meiotic division but on exiting meiotic arrest, a dramatic burst of Oct-4 expression occurs suggesting that Oct-4 may have a role in the oocyte growth and/or acquisition of meiotic competence (Pesce *et al*, 1998). If this is case, it may be expected that oocytes null for Oct-4 protein may be non viable and unable to complete meiotic maturation and/or undergo successful fertilisation.

The objective of this study was to investigate the role of Oct-4 in the mammalian germ line using a Cre-LoxP approach. Two tissue specific Cre expression lines were used, TNAP-Cre (Lomeli *et al*, 2000) and ZP3-Cre (Lewandoski *et al*, 1997) that expressed Cre in germ cells and during oogenesis respectively. In combination with an Oct-4 floxed line and a null Oct-4 line, mice were generated of a TNAP- and ZP3- $Cre^{+/-}$ Oct-$4^{-/loxP}$ genotype. These mice were analysed for the consequences of Cre mediated deletion at the floxed Oct-4 locus in germ cells and in oocytes. Unfortunately complications regarding the temporal and spatial expression of the TNAP-Cre expression line prevented definitive conclusions being reached on the role of Oct-4 in germ cells.
The pattern of TNAP-Cre and ZP3-Cre mediated deletion was inferred from the use of two reporter lines, sGFP and R26R, to indicate the Cre spatial and temporal deletion

pattern. This was due to the fact that a reporter cassette was not incorporated into the floxed Oct-4 locus. However, it has been that reported the recombination efficiencies for Cre recombinase can vary between target loci (Nagy 2000); Muller, U. 1998) such that Cre mediated deletion by TNAP-Cre and ZP3-Cre at the reporter loci may not reflect that at the floxed Oct-4 locus.

5.1 Consequences of TNAP-Cre mediated deletion at the floxed Oct-4 locus.

Activation of dormant reporter lines showed that ectopic TNAP-Cre activity was detected in preimplantation embryos as opposed to being confined to the germ cells later during development. Analysis of TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ adult mice that were expected to lack Oct-4 in their germ cells were fertile and E12.5 embryos of the same genotype had no severe apparent defects in germ cells numbers in their genital ridges. Pups born from these animals exhibited a mosaic pattern of inheritance of the recombined/non-recombined allele indicating that Cre mediated deletion occurred post fertilisation in these pups and not as a result of deletion in the germ cells. No pups were born that could be attributed to originate from an Oct-4 null germ cell. In conclusion mosaic ectopic TNAP-Cre mediated deletion in the early embryo is likely to account for recombination at the floxed Oct-4 target allele. Furthermore, no Cre recombinase was detected in genital ridges from E12.5 TNAP-Cre$^{+/-}$ embryos. These results are discussed below.

TNAP-Cre exhibited ectopic mediated deletion in the early embryo onwards for both reporter line studies. The presence of mosaic and non mosaic embryos in the same litter suggest stochastic differences in TNAP-Cre activity between embryos in the same litter that are independent of the original parental allele. It is well reported that differences in genetic background can have widely differing effects on the phenotype observed for both naturally occurring and targeted mutations (Muller, 1999). The two

genetic backgrounds used were 129 and C57BL/6 for establishment of TNAP-Cre lines and animals were bred for only three generations (N3). Mixing of genetic backgrounds was required to breed TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice. The published TNAP-Cre mouse line (Lomeli *et al*, 2000) was on a 129 ICR M background. The differences in genetic background may account for the ectopic activity of the TNAP-Cre line used in this study due to the presence or absence of allelic variant of genes, referred to as modifier loci (Muller, 1999). Lomeli and colleagues did report a degree of ectopic TNAP-Cre mediated deletion, this manifested in mosaic expression throughout the embryo which was enhanced if the *TNAP-Cre* allele was maternal in origin (Lomeli *et al*, 2000). 50% of embryos analysed did exhibit restriction of reporter line expression in the PGCs at E9.5 – 10.5 (Lomeli *et al*, 2000).

From the reporter studies it was clear that the TNAP-Cre line exhibited early ectopic mediated deletion but it was not apparent whether or not Cre was expressed in the germ cells. RT-PCR analysis of E12.5 genital ridges from TNAP-Cre$^{+/-}$ embryos showed that no Cre recombinase mRNA was detectable. Given that approximately 20 000 germ cells were present in each genital ridge and the RNA was made from eight pooled genital ridges there might not have been enough Cre cDNA to amplify and visualise for this reaction even if the Cre mRNA was at a low level. Also Lomeli and colleagues did not state whether or not they had analysed the germ cells at this or any point for actual Cre mRNA, instead they used reporter line studies to assay for activity (Lomeli *et al*, 2000). This approach would only identify when Cre mediated deletion first occurred and does not relay information as to whether or not Cre was expressed, and causes recombination, later during development. Analysis of the TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice would support Cre not being expressed in the genital ridges. Alkaline phosphatase staining of germ cells in E12.5 genital ridges from TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos demonstrated there were no gross abnormalities in germ cell number and also these animals showed normal levels of fertility. The efficiency of recombination by the

TNAP-Cre line was found to be approximately 60% at the Z/AP reporter loci (Lomeli et al, 2000). If Cre was expressed earlier but expression not maintained by PGCs in the genital ridge, it would be likely that if sufficient numbers of non recombined germ cells remained they may be able to compensate for any loss of null Oct-4 germ cells since whilst migrating and on reaching the genital ridge they continue dividing; mitotic division ceases at E16.5. The inheritance pattern of the floxed Oct-4 allele (either recombined or non recombined) in pups born from TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ adult mice showed that if recombination did occur it occurred in a mosaic pattern and only where TNAP-Cre had also been inherited. This suggests that the recombination at the floxed Oct-4 locus did not occur in the germ cells but was due to early ectopic Cre mediated deletion in the embryo.

A further observation regarding the TNAP-Cre line was that maternal inheritance of the TNAP-Cre allele in TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos resulted in a significant loss of some embryos of this genotype. Oct-4 is absolutely required in the ICM (Nichols et al, 1998) and the epiblast for normal development (unpublished, J Nichols). Given the potentially variable Cre mediated deletion activity of the TNAP-Cre line it is conceivable that in embryos where TNAP-Cre mediated deletion occurred throughout the ICM and/or epiblast these embryos would be lost which may account for the fraction of TNAP-Cre$^{+/-}$ Oct-4$^{-/loxP}$ embryos that were not present. Lomeli and colleagues did report that the maternal contribution of the TNAP-Cre allele appeared to result in an increased incident of mosaic expression for the Z/AP reporter allele and they attributed this to a maternal effect; either the presence of TNAP-Cre RNA in the oocyte or different specificity of the maternal *TNAP-Cre* allele (Lomeli *et al*, 2000). Given the consistent early ectopic activity of the TNAP-Cre line observed for both maternal and paternal inheritance of the *TNAP-Cre* allele in this study it was not possible to ascertain if this was the same maternal effect they had observed. Furthermore, TNAP-Cre

mediated deletion may vary between target loci such that deletion at reporter loci may not reflect that at the floxed Oct-4 locus.

5.2 Consequences of ZP3-Cre mediated deletion at the floxed Oct-4 locus.

Activation of dormant reporter lines showed that ZP3-Cre mediated deletion was found to be specifically confined to developing oocytes and was detectable in primary oocytes onwards. Pups born from ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ females, that theoretically lacked Oct-4 in their oocytes, always inherited the recombined Oct-4 allele in a Mendelian fashion. This suggested that recombination at the floxed Oct-4 locus had occurred in the oocyte or possibly in the single cell embryo; raising the possibility that Oct-4 is not required in oocytes. In mutant animals it was not possible to deduce whether the oocytes were actually deficient in Oct-4 protein and given the fact that all oocytes of the correct genotype had undergone recombination it was also conceivable that the timing of the excision may have occurred post the burst of Oct-4 expression in oocytes as they exit meiotic arrest. These results and reasons for late excision and lack of phenotype are discussed below.

For the ZP3-Cre line, both reporter line studies indicated that ZP3-Cre mediated deletion occurred in the expected pattern (Lewandoski *et al*, 1996) and was clearly detected in oocytes from the early stages of meiotic arrest onwards coinciding with the published expression pattern of ZP3 (Phillpott *et al*, 1987). Reporter line studies and generation of ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice required mixing of genetic backgrounds but no apparent ectopic activity of the ZP3-Cre allele was detectable for the reported line studies unlike for TNAP-Cre. ZP3-Cre$^{+/-}$ Oct-4$^{-/loxP}$ mice were fertile, but produced smaller than average litter sizes. This can be attributed to the presence of *ZP3-Cre* allele since heterozygous ZP3-Cre animals tend to have smaller than average litter sizes (unpublished observation, B. Knowles). Analysis of genotypes of pups born from ZP3-

$Cre^{+/-}$ $Oct\text{-}4^{-/loxP}$ female mice showed that the floxed Oct-4 allele where inherited was always recombined and this was independent of the inheritance of the ZP3-Cre allele. This would indicate that ZP3-Cre mediated recombination at the floxed Oct-4 locus had occurred prior to fertilisation or in the one cell embryo but the exact time point during oogenesis when deletion had occurred was not known. The reporter line studies suggested that ZP3-Cre mediated deletion occurred from the early stages of meiotic arrest but as no reporter was activated on recombination at the floxed Oct-4 locus the exact timing of recombination at the locus was not known. To attempt to elucidate whether Oct-4 protein was present or absent in such oocytes α-Oct-4 antibodies were used to stained sectioned or whole oocytes. These experiments were inconclusive; furthermore attempts to perform Oct-4 *in situ* hybridisation on ovary sections and whole mount were also unsuccessful. One way of establishing when recombination had occurred at the floxed Oct-4 locus would be to use an *in situ* PCR approach, this was not actually attempted.

Pups born from ZP3-$Cre^{+/-}$ $Oct\text{-}4^{-/loxP}$ female either inherited an $Oct\text{-}4^{-}$ or $Oct\text{-}4^{floxed\ R}$ allele. There appeared to be a discrepancy in the numbers of null alleles *versus* floxed recombined alleles inherited. It was expected this would be in a 1:1 ratio since oocytes with null allele are viable in heterozygous mice due to the presence of maternal mRNA from the wildtype Oct-4 allele. Given that recombination at the floxed Oct-4 locus at a given time point would result in an oocyte (4N, 2N or 1N depending when deletion occurred) being null for Oct-4 potentially being lost the sister oocyte that had inherited the null allele would be expected to be lost also hence a 1:1 ratio would be obtained (see Figures 4.1 and 4.2). The small numbers of pups analysed or PCR errors may account for this artifact. It was not possible to deduce whether or not oocytes from ZP3-$Cre^{+/-}$ $Oct\text{-}4^{-/loxP}$ female mice were null for Oct-4 so it could not be ascertained if the deletion at the floxed Oct-4 locus had occurred too late or not i.e. prior to the burst in Oct-4 expression first occurring in oocytes of approximately 50μm in diameter as they exit

meiotic arrest. If the deletion had occurred later than expected, residual maternal Oct-4 mRNA and/or protein may have been sufficient to carry the oocyte through to fertilisation.

Reasons for late deletion are unclear but one possibility could be due to physical masking of the Oct-4 locus during meiotic arrest. Previously it has been shown that *in vitro* extinction of Oct-4 expression is associated with changes in methylation, chromatin structure and transcriptional activity in the 5′ upstream regulatory region (Ben-Shushan. E *et al*, 1993). More recently Gidekel and colleagues have shown that *in vivo* the Oct-4 gene harbours a *cis* specific demodification element that includes the PE sequence. This element resulted in demethylation in EC cells where the sequence was initially methylated and protected the surrounding local region from *de novo* methylation in post implantation embryos (Gidekel and Bergman, 2002). This element provides a mechanism that ensures undermethylation of genes that are required to be expressed at a time when *de novo* methylation is occurring. It has been proposed that during oogenesis the PE demethylation element may play a role in protecting the Oct-4 gene from undergoing *de novo* methylation at E15.5 thereby allowing its later re-expression as oocytes exit meiotic arrest. This may be mediated by the binding of specific trans-acting factors to the PE element that protect the surrounding sequences from global methylation. The extent of binding of such unidentified factors is unclear but they could potentially interfere with Cre mediated recombination at the floxed Oct-4 locus and account for delayed deletion.

5.3 *Considerations for tissue specific targeted gene inactivation using the Cre-LoxP system.*

This project has further reinforced some of the problems that are becoming more apparent with the Cre-LoxP system for tissue specific targeted gene inactivation. As

with the TNAP-Cre line expression of Cre recombinase under the control of an endogenous promoter does not always reflect the expected expression pattern and this can bring about serious complications in phenotype analysis. For instance when the germ cell-specific phosphoglycerate kinase 2 (Pgk2) promoter was used to drive Cre expression in spermatocytes and spermatids ectopic expression was detected in other tissues during embryogenesis and in non testis tissue in adult mice (Bhullar *et al.* 2001). Also ectopic expression of Cre was detected in the cartilaginous portions of the ribs where Cre expression was directed by the proximal Pax3 promoter which is usually restricted to the neural crest (Li *et al.* 2000).

Mosaicism is a critical factor in the majority of tissue specific gene inactivations since recombination efficiencies are not 100% (Kwan 2002). As a result deletion of the conditional allele in only a subset of target cells occurs creating a situation where the wildtype population of cells may potentially obscure and/or mask the inactivation phenotype. In order to compensate for this problem the usual approach is to trace/distinguish the two cell populations at the cellular level after Cre mediated deletion. This can be achieved by using *in situ* hybridisation and/or immunostaining to detect the specific mRNA transcript or protein respectively. A more direct and reliable way of circumventing this problem is to incorporate a reporter gene into the floxed target gene that is activated upon recombination. However, this is also problematic because insertion of exogenous sequences can disrupt regulatory regions of the gene and other non related genes which may complicate phenotypic interpretation.

Relying on an independent reporter line to reflect recombination at the target floxed is not always accurate since recombination efficiencies can vary between target loci. For instance marked differences in recombination efficiencies for different floxed target alleles were found where Cre-ER^{T} was expressed from the R26R locus *in vivo* (Vooijs *et al*, 2001). Difference in target allele activation frequencies may be related to local

chromatin structures which may affect accessibility (Muller, 1999). For both TNAP-Cre and ZP3-Cre lines both reporter lines gave similar patterns of Cre mediated deletion activity.

As with all targeted mouse mutations the genetic background can play an important role in the resultant phenotype (Muller, 1999). For example targeted inactivation of the epidermal growth factor (EGF) receptor has widely different phenotypic consequences depending on the genetic background of the mouse line. Lethality occurs at E7.5 on a CF-1 background, midgestation on a 129/Sv background, perinatally on a CD-1 background and postnatal lethality on a 129/Sv x C57BL/6 background (Sibilia *et al.* 1995; Threadgill *et al.* 1995). No such published examples exist of differences in Cre recombinase activity subject to genetic background, but given that the issue of background variability in transgenic lines has gained wide spread attention recently it should be considered (Muller, 1999).

5.4 Conclusions and future perspectives

The use of the Cre-LoxP system for tissue specific targeted gene inactivation, though conceptually straightforward, requires rigorous testing of both the Cre line and floxed target allele to avoid complications in phenotypic interpretation. This can be circumvented to some extent by incorporating a reporter construct at the floxed target locus which is activated upon Cre mediated recombination. The consequences of introducing such a cassette into the Oct-4 locus are unknown and if there was any disruption in the expression of Oct-4 it would be likely the embryo would be non viable due to loss of pluripotency in the ICM. Failing incorporation of a reporter construct, detection of the loss of the protein in those cells that have undergone recombination with an appropriate antibody or *in situ* RNA probe can be carried out. Both of the studies here relied on the latter, neither of which gave conclusive results. TNAP-Cre

mediated deletion could not be detected in germ cells and this was complicated by the fact that early ectopic Cre mediated deletion did occur in the early preimplantation embryo. The reasons for this are likely to be due to stochastic differences at the *TNAP-Cre* allele compounded by effects of genetic background. ZP3-Cre mediated deletion did occur such that all pups born that had inherited the floxed Oct-4 allele had undergone recombination. This suggested that recombination at the floxed Oct-4 locus had occurred in the oocyte or possibly in the single cell embryo. It was not possible to elucidate if Oct-4 protein was present in such oocytes. It was also conceivable that excision at the floxed Oct-4 locus had occurred too late, post the burst of Oct-4 expression as oocytes exit meiotic arrest and as a result maternal mRNA was sufficient to carry the oocyte through to fertilisation. One possible reason for excision occurring late could be due to physical masking of the Oct-4 locus during meiotic arrest. If this was the case, a Cre-LoxP approach to address the original question of the role of the burst of Oct-4 in oocytes as they exit meiotic arrest may not be a viable approach. Recently endogenous Oct-4 and c-*mos* gene were successfully inactivated in oocytes using short interfering RNAs (Kim *et al.* 2002) and this could be another approach considered in the future.

Chapter 6: References

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